REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
04/02/2016	Final	04-11-2013 to 04-02-2016

4. TITLE AND SUBTITLE
Applications and Methods for Continuous Monitoring of Physiological Chemistry

5a. CONTRACT NUMBER
W31P4Q-14-C-0015

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
David A. Johnson, Peter Petillo, Daniel Aillon, Mark Richter and Philip Gao

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Pinnacle Technology, Inc.
2721 Oregon Street
Lawrence, KS 66046-4947

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Army Contracting Command - Redstone
Redstone Arsenal, AL 35898-5280

10. SPONSOR/MONITOR'S ACRONYM(S)
DARPA

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT

13. SUPPLEMENTARY NOTES

14. ABSTRACT
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15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			David A Johnson
					19b. TELEPHONE NUMBER *(Include area code)*
U	UU	UU	UU	69	785-832-8866

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std. Z39.18

DARPA Phase II Final Contractor Report
Contract W31P4Q-14-C-0015
Topic No. SB12A-003
February 4, 2016

Applications and Methods for Continuous Monitoring of Physiological Chemistry

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Final Technical Report

Applications and Methods for Continuous Monitoring of Physiological Chemistry

For the Period:

November 5, 2013 to February 4, 2016

Sponsored By:

Defense Advanced Research Project Agency (DoD)

(Controlling DARPA Office)

ARPA Order Nr. HR001132946-BASIC

Issued by U.S. Army Aviation and Missile Command Under
Contract No. W31P4Q-14-C-0015

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ABSTRACT

The overall goal of this project was to develop a platform methodology to clone, evolve, purify and stabilize high activity oxidase enzymes that are suitable for point-of-care diagnostic and human biosensor applications. The general approach was to add fusion proteins to increase enzyme stability without compromising enzyme activity. To demonstrate feasibility, oxidase enzymes that recognize lactate and histamine were improved, and an effort was made to develop a cortisol oxidase from cholesterol oxidase. These studies set the stage for a platform methodology to create biorecognition elements that are suitable for a wide range of point-of-care diagnostic devices and biosensors, and this will greatly enhance the tools available in the development, clinical, first responder and battlefield arenas.

TABLE OF CONTENTS

Table of Figures

List of Tables

1 EXECUTIVE SUMMARY

1.1 Introduction

Point of care (POC) diagnostic devices are well established, but significant growth opportunities remain. POC testing includes all screening and testing that occurs outside of the main hospital laboratory. In the last 10 – 20 years, more testing is being done at the patients' bedsides in hospitals, in doctors' office laboratories, outpatient clinics, emergency rooms and intensive critical care facilities. However, in most cases, these POC devices are still large, dominated by glucose monitoring, blood chemistry and electrolytes, cardiac markers, and they require skilled operators. The overall challenge is to miniaturize the POC devices, decrease cost, allow the tests to be performed by minimally trained individuals, provide immediate results, and increase the types of tests available.

Many of the new POC devices under development are for the immediate field assessment of nucleic acids, proteins, and small molecules. Examples include electrode arrays[1, 2] microfluidics,[3] paper based microfluidics,[4, 5] biosensors,[6] and others. They use a wide variety of detection schemes including electrochemical,[7] colorimetric,[8,9] chemiluminescence,[10] and electrochemiluminescence.[11, 12] One key component of many of these devices is the biological recognition element. Stable off-the-shelf biological recognition elements available in a form that can be readily incorporated into POC devices will be essential for wide spread use and deployment. This is currently a major operational gap for the POC devices to be used for highly multiplexed reconfigurable diagnostic platforms or in low complexity instruments.

In general, the problem has been that, with the exception of glucose oxidase, high purity, high selectivity, high stability and high activity enzymes are not available,[13] and those that are available, are not produced in the United States. The current commercial supply of oxidase enzymes suffers from the following problems:

- The range of commercially available oxidase enzymes is limited for biomedical applications.
- Other than glucose oxidase, oxidase enzymes in general have low unit activity and varying stabilities.
- The available oxidases are primarily derived from natural sources, and they have widely varying properties depending on the source, time on the shelf, manufacturing process, etc. Since the unit activity of enzymes decreases with age, different batches from the same source can have widely variable characteristics. There is tremendous company to company variability.
- Other than glucose oxidase, the majority of oxidase enzymes suitable for biosensors originate from four Japanese companies; Yamasa, Toyobo, Sekisui, and AsahiKesei.
- The enzymes are repackaged (sometimes multiple times) prior to resale in the United States. Continuous handling of an enzyme can compromise its unit activity, and create lot-to-lot variability, which further complicates the use of the enzyme for biosensor applications.

The overall goal of this project was to show that a platform methodology could be designed to clone, evolve and stabilize high activity oxidase enzymes that are suitable for POCs and human biosensor applications. This was accomplished by demonstrating that the addition of fusion proteins to wild type oxidase enzymes can stabilize the enzyme without compromising catalytic

activity. This project leveraged modern protein engineering concepts and protocols to provide oxidase enzymes with improved cofactor residency and improved stability profiles.

This effort was very successfully in some ways and not successful in others. We have a lactate oxidase with great properties and stability. It has yielded biosensors with good performance characteristics and there is interest from outside parties in lactate POC devices. Histamine oxidase development did not yield an enzyme that works well in a POC device, but there are opportunities for continued developed. There was nothing we tried that yielded a cortisol oxidase with characteristics that would be useful to an end user. However, as part of this research, several new cholesterol oxidases were developed with unique characteristics.

1.2 Methods

Guided by past efforts of this group and the literature, we employed modern molecular biology to re-engineer and evolve an existing oxidase into an enzyme that is specific for cortisol, and to improve the enzymatic performance and stability for oxidases specific to lactate and histamine. We are developing a paradigm for re-engineering enzymes with the selectivity and stability profiles needed for biosensing applications. This approach to enzyme re-engineering is unique and innovative in the diagnostic field and positively impacts the development and manufacturing of devices for novel applications.

The project team has considerable experience in the large scale expression, purification, folding, lyophilization, and formulation of proteins. This experience has been leveraged to determine the most efficient protocols for producing lactate oxidase, and histamine oxidase in quantities and purities suitable for sale and use in diagnostic devices.

Finally, we used Pinnacle's biosensors both as a product and test platform to verify the performance characteristics of the enzymes when used in diagnostic device fabrication.

1.3 Results

This project had three primary objectives:

1. Engineer a cortisol oxidase enzyme suitable for use in diagnostic devices
2. Large scale production and formulation of lactate oxidase, histamine oxidase and the newly engineered cortisol oxidase.
3. *In vitro* and *in vivo* testing of the enzymes to verify their efficacy as part of a diagnostic device.

1.3.a Evolution of Cortisol Oxidase

Despite extensive effort the cortisol directed evolution screens were consistently negative. We implemented an alternative evolutionary strategy of generating mutant libraries using error-prone PCR and DNA shuffling then directly screening for enhanced cortisol oxidation activity in a ninety-six well format. This approach is similar to that used by Minagawa et al. in isolating mutants of the lactate oxidase. Note that the random nature of the experiment means that multiple experiments performed in exactly the same manner can give dramatically different results. This was also not effective. A random, FACS-based mutant selection method was also tried without success.

In all, we screened thousands of wells, tweaked the processes in numerous ways, and followed a number of false trails. In the end, we found a number of cholesterol oxidase variants that may prove to be very valuable for diagnostic devices and other applications, but we were not able to produce a form of the enzyme that showed significantly more activity and specificity to cortisol then the wild type cholesterol oxidase. We did identify 18 new mutations that may confer cortisol oxidase activity to the construct. These are summarized in the results section, and may lead to development of a viable cortisol oxidase in future studies.

1.3.b Large Scale Production of Lactate Oxidase, Histamine Oxidase and Cortisol Oxidase

Lactate Oxidase:

A large-scale production protocol for MBP-LOx was determined. MBP-LOx can be produced in large scale by using an enhanced Luria broth protocol followed by refolding of the resultant inclusion bodies. Based on our activity experiments on a biosensor, we identified three sets of conditions that provide well-formulated MBP-LOx enzyme, 70% sucrose, 90% sucrose and 90% trehalose. Of these, the 90% sucrose formulation provided the best overall activity, especially as a function of time. This was the formulation used for the final MBP-LOx protocol. We also performed lyophilization studies of MBP-LOx constructs in a variety of glycerol concentrations. Despite the universal deployment of glycerol in protein biochemistry as a cryoprotectant, its use, in this case, compromises the activity of MBP-LOx when used on a biosensor.

In further work, we improved the base protocol to reduce batch-to-batch, assay-to-assay, and technician-to-technician variability. As a final test, two 1 liter lots of MBP-Lactate oxidase were manufactured. Both lots exceed targets (protein concentration & activity) set in early 2015. The lactate biosensors produced from both enzyme lots produced responses in the desired sensor range. Multiple assays were performed on the enzyme lots to examine the consistency of the assays. Biosensors produced from these lots show excellent in vitro response, and the assay consistency was acceptable. The results are shown in Figure 16, Figure 17 and Table 5.

Histamine Oxidase:

The team achieved excellent large scale production results for both MBP-zmPOA and MBP-osPOA using enhanced Luria broth (described in 4.2a). As an example, we were able to produce almost 22 mg of MBP-zmPOA from 1 liter of soluble expression. This is the first example of soluble expression of this material at reasonable levels. Historically, expression levels were about 0.25 mg/L. Thus, this constitutes an almost 100-fold improvement in expression efficiency.

Cortisol Oxidase:

Since we were unable to produce an oxidase with sufficient specificity and activity toward cortisol, we did not develop production/lyophilization protocols. It is very reasonable to believe that the basic procedures developed for lactate oxidase and histamine oxidase will be an excellent starting place for cortisol oxidase once the construct is in place.

1.3.c In Vitro and In Vivo Testing of the Enzymes Developed

We had proposed to use biosensors as the testing device to determine if the enzymes developed had the characteristics necessary to be used in POC devices.

Lactate Oxidase:

Lactate biosensors can be routinely manufactured using the MBP-lactate oxidase enzyme (90% sucrose formulation). Sensitivity testing of the MBP-lactate oxidase biosensors found that they meet or exceed the performance biosensors made with commercial (Sekisui) lactate oxidase in terms of limit of detection, sensitivity and linearity. Shelf-life stability testing under different storage conditions showed that in a test of 80 CNS diagnostic devices good to great stability was observed in all storage conditions. In particular, Figure 25 shows excellent stability over an 11 week period. This is significantly greater than that seen with available commercial forms of lactate oxidase, and this result was obtained with repeated sensor testing. That is, the sensor was repeatedly hydrated and dehydrated – a process known to stress the immobilized enzyme.

Histamine Oxidase:

We demonstrated that the fusion proteins can be used to add stability to the oxidases without compromising activity. However, the histamine oxidases developed to date are not active enough in immobilized form for use in a biosensor of the type normally produced by Pinnacle. These oxidases may be very valuable in other POC applications.

Cortisol Oxidase:

The response to cortisol remained low throughout the project. Cortisol biosensors were constructed, but as was the case with histamine, there is some direct action between the cortisol and platinum substrate. Some preliminary subcutaneous testing was performed (Figure 41, and Figure 42), but once it was clear that the primary response was likely due to direct cortisol/platinum interaction, no additional *in vivo* testing was carried out.

1.4 Discussion and Future Plans

As part of its immediate future plans for this project – there are four key commercialization projects on-going and two research projects.

1. Pinnacle is transitioning the lactate enzyme developed under this contract to use in its biosensors.

2. In December 2015, Pinnacle signed a Service Agreement with a Fortune 100 company to try using the lactate enzyme on the Company's POC device. The first PO against that agreement for R&D has been completed and the initial research was very successful.

3. Another major Company is in discussion with Pinnacle regarding using the lactate enzyme in a POC device. We expect to have a signed agreement by mid-2016.

4. To date, a large selection of enzymes have been derived as part of this project. To make these enzymes widely available for use in point-of-care devices, Peter Petillo, former CSO of Pinnacle and PI on this project, is in the process of setting up a spin-out company devoted solely to the continued development and sale of these, and other, enzymes. The proper legal agreements between Pinnacle and this new entity are being negotiated. To date enzyme constructs derived from this effort include: wt-osPOA, MBP-osPOA, SUMO-osPOA, wt zmPOA, MBP-zm-POA, SUMO-zmPOA, wt-LOX, MBP –LOX, SUMO-LOX,

MBP-LOX-6m, SUMO-LOX-6m, wt-COX1, MBP-COX1, SUMO-COX1, wt-COX2, MBP-COX2, SUMO-COX2, wt-PutOx, MBP-PutOx.

5. The histamine enzymes developed to date may be suitable for point-of-care devices and other applications but do not have the characteristics necessary for a functional biosensor. We have identified a histamine oxidoreductase [14] that looked to be acting as an oxidase. This was purchased, sub-cloned, sequenced, and expressed. This enzyme is very specific to histamine, but the activity is low, and it does not produce peroxide directly. We are still optimistic about this construct's potential moving forward. Our investigations will continue.

6. As part of the effort to develop an oxidase that is specific and active toward cortisol, several cholesterol oxidases were developed that are significantly more thermally stable, more resistant to denaturation by organic solvents and more resistant to denaturation by detergents than the *Streptomyces sp.* enzyme.

2 INTRODUCTION

It is difficult to continuously monitor biomarkers in real time. In general, samples are collected, shipped, analyzed and the results returned. Continuous measurement of biomarkers including immunological signatures or particular subsets of proteins, nucleic acids, or small molecules could provide diagnostics for specific diseases, traumatic injury, or an indication of a shift in baseline health. Biomarkers need to be measured in real time with minimal, or no, post-processing. Point of care (POC) diagnostic devices are well established, but significant growth opportunities remain. POC testing includes all screening and testing that occurs outside of the main hospital laboratory. In the last 10 – 20 years, more testing is being done at the patients' bedsides in hospitals, in doctors' office laboratories, outpatient clinics, emergency rooms and intensive critical care facilities. However, in most cases, these POC devices are still large, dominated by glucose monitoring, blood chemistry and electrolytes, cardiac markers, and they require skilled operators. The overall challenge is to miniaturize the POC devices, decrease cost, allow the tests to be performed by minimally trained individuals, provide immediate results, and increase the types of tests available.

Many of the new POC devices under development are for the immediate field assessment of nucleic acids, proteins, and small molecules. Examples include electrode arrays[1, 2] microfluidics,[3] paper based microfluidics,[4, 5] biosensors,[6] and others. They use a wide variety of detection schemes including electrochemical,[7] colorimetric,[8,9] chemiluminescence,[10] and electrochemiluminescence.[11, 12] One key component of many of these devices is the biological recognition element. Stable off-the-shelf biological recognition elements available in a form that can be readily incorporated into POC devices will be essential for wide spread use and deployment. This is currently a major operational gap for the POC devices to be used for highly multiplexed reconfigurable diagnostic platforms or in low complexity instruments.

The project team of Pinnacle Technology and the University of Kansas' has considerable experience in cloning, expression, purification and stabilization of oxidase enzymes that will improve existing, and enable new, human biosensing applications. We used Pinnacle's ability to rapidly produce prototype biosensors as the readout as to whether a protein preparation is suitable for biosensing applications. We tested the use of fusion proteins as a general method of stabilizing oxidase enzymes.

Pinnacle has developed and sells different biosensors into the preclinical market. Biosensors employ an enzyme, typically from the oxidase family, as the sensor's biorecognition element. While there are many other aspects of a biosensor's final design that are important and require optimization (e.g., inner and outer membranes, the matrix in which the enzyme is embedded, loading, large scale production), none are as vital as a properly folded enzyme with suitable activity and stability profiles.[13, 15] To this end, we proposed to develop a platform methodology to screen, clone, evolve and stabilize oxidase enzymes for the measurement of metabolic biomarkers. In collaboration with the University of Kansas, Pinnacle will develop these enzymes to enhance Pinnacle's line of biosensors and biosensor arrays for both animal and human use, and for direct sale to other entities to manufacture biosensors and other products for human monitoring. The enzymes produced will be of use in amperometric, spectrophotometric, and other methods that rely on biorecognition elements.

To date, glucose biosensors are the only biosensors routinely used in humans to continuously measure a biomarker in real-time.[16] Due to the excellent stability and activity of glucose oxidase

(often referred to as "an ideal enzyme"),[17] reliable glucose biosensors for clinical diagnostic monitoring can be manufactured.[18] This has translated to the widespread use of biosensors for glucose monitoring. As an indication of the market potential for high quality biosensors, the glucometer market is anticipated to be $6 Billion by 2015.[19] This enzyme has been thoroughly characterized and has proven to be amongst the most active and most stable member of this enzyme family. Furthermore, the enzyme's kinetic parameters are such that it is ideally suited for biosensor applications.[17, 18]

Oxidase enzymes are used in biosensor construction because family members are known to process a variety of analytes that are important to human health.[20, 21] Further, for the oxidases typically used in amperometric biosensors, the by-product of the enzyme, hydrogen peroxide, is produced in a one-to-one ratio to the analyte present. It is important to note that oxidases only require O_2 to regenerate and do not require the presence of additional substrates beyond the analyte of interest. Oxidases have been shown to be effective as the biorecognition element in the development and commercialization of biosensors.[22] Oxidase enzymes employ a range of cofactors to support their redox cycles, and the absence of the co-factor renders the enzyme catalytically inert. In this project, we focused on oxidase enzymes that employ flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) as co-factors. Pinnacle has already developed intellectual property (IP) (patent PCT/US11/051193) that provides a general solution for the use of cofactors to enhance enzyme unit activity, and therefore the sensitivity of the biosensor.

A real challenge in the development of new biosensors and POC devices that use oxidase enzymes is the availability of enzymes. In general, the problem has been that, with the exception of glucose oxidase, high purity, high selectivity, high stability and high activity enzymes are not available,[13] and those that are available, are not produced in the United States. The current commercial supply of oxidase enzymes suffers from the following problems:

- The range of commercially available oxidase enzymes is limited for biomedical applications.
- Other than glucose oxidase, oxidase enzymes in general have low unit activity and varying stabilities.
- The available oxidases are primarily derived from natural sources, and they have widely varying properties depending on the source, time on the shelf, manufacturing process, etc. Since the unit activity of enzymes decreases with age, different batches from the same source can have widely variable characteristics. There is tremendous company to company variability.
- Other than glucose oxidase, the majority of oxidase enzymes suitable for biosensors originate from four Japanese companies; Yamasa, Toyobo, Sekisui, and AsahiKesei.
- The enzymes are repackaged (sometimes multiple times) prior to resale in the United States. Continuous handling of an enzyme can compromise its unit activity, and create lot-to-lot variability, which further complicates the use of the enzyme for biosensor applications.

An attractive feature of this enzyme class, is that oxidase enzymes, most especially those that are FAD and FMN dependent, can be readily over-expressed in *E. coli*, thereby facilitating the production of the wild-type enzyme on a useful scale. In turn, this allows standard molecular biology techniques to be used to modify existing sequences to improve a particular enzyme's stability and, in some cases, to dramatically improve the enzyme's selectivity and catalytic activity profiles.

Finally, many oxidase genes are part of fundamental biosynthetic pathways, especially in bacteria and yeast. Thus, these genes can be manipulated with selective pressure to evolve existing oxidase genes into new enzymes with specifically tailored selectivity. This has been shown in the literature, and will form the basis for much of the II work related to this proposal. Good Laboratory Practice (GLP) compliant recombinant enzyme production, using the techniques outlined in this proposal, will result in a wider range of biomarker targets, higher stability, greatly improved shelf life, and consistent properties from lot-to-lot. The ability to produce custom-made enzymes capable of processing analytes for which no enzyme presently exists ultimately expands the analytes that may be leveraged for human sensing applications. One can envision that custom crafted sensing arrays, based on a group of evolved oxidase enzymes, would aid in point of care diagnostics and numerous other applications.

The overall goal of this project was to show that a platform methodology could be designed to clone, evolve and stabilize high activity oxidase enzymes that are suitable for POCs and human biosensor applications. This was accomplished by demonstrating that the addition of fusion proteins to wild type oxidase enzymes can stabilize the enzyme without compromising catalytic activity. This project leveraged modern protein engineering concepts and protocols to provide oxidase enzymes with improved cofactor residency and improved stability profiles.

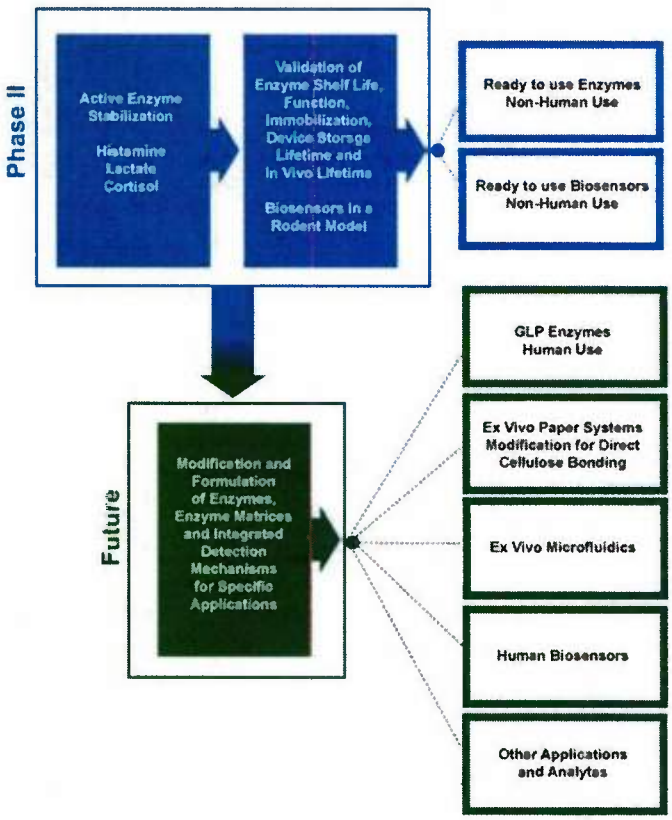
Pinnacle has already developed intellectual property (patent PCT/US11/051193) that provides a general solution for the use of cofactors to enhance enzyme unit activity on a biosensor, and therefore the sensitivity of the biosensor. Pinnacle has also begun testing the enhancements that fusion epitopes add to the properties of a wild type enzyme. For oxidase enzymes, these fusion proteins have been shown to enhance unit activity without compromising, and in some cases enhancing, catalytic activity as measured by the Michaelis constant (K_M) and the unimolecular rate constant (k_{cat}). Preliminary testing was carried out on glutamate oxidase, nicotine oxidase and putrescine oxidase. We hypothesize that this approach will be applicable to most oxidase enzymes.

In the first Phase of this project, the team demonstrated that the addition of fusion proteins to a wild type oxidase enzyme can stabilize the enzyme without compromising its catalytic activity. Fusion proteins, in the context of this proposal, are the combination of epitope tags added to the N- or C-terminus of a recombinant protein. Historically, these tags were introduced to improve protein recovery by improving the over-expression, isolation, solubilization and purification of a recombinant protein. The epitope tags range from short poly-peptides to proteins of several hundred amino acids. These tags include (but are not limited to) MBP (maltose binding protein, 396 amino acids), GST (glutathione S-transferase, 201 amino acids), and Gro-EL (192 amino acids). These epitope tags are usually attached to the wild type protein sequence via a short linker, which often includes a cleavage site to allow for separation of two proteins if needed. In addition to the traditional advantages resulting from fusion protein assemblies, we have found that several common fusion protein epitopes may also confer added stability to the wild type protein. This is especially true for oxidases.

Before additional steps can be taken to manufacture POC devices that have a minimum shelf-life of 3 months and a minimum *in vivo* life of 2 weeks, we must have stable enzymes. This project's target was to validate the hypothesis that oxidase enzyme stability can be substantially increased with fusion proteins without compromising catalytic activity, and also to demonstrate that the ability to easily clone these oxidase enzymes will ultimately reduce the batch to batch variability and natural source variability that currently exists.

During Phase I, the team demonstrated that oxidases selective for lactate and histamine can be cloned and expressed on a useful scale by over-expression in *E. coli*, improved by the addition of fusion proteins, and synthesized to a form that is amenable to immobilization while maintaining activity. In Phase II, we continued to develop clones of lactate oxidase and histamine oxidase that have a long shelf life, long active life and high activity. The effort for lactate oxidase was very successful. The developed oxidase is as active, and much more stable, than anything currently available commercially. This enzyme is suitable for use in our own biosensors as well as other point-of-care devices. The histamine oxidase developed is a significant improvement over anything commercially available. It is not suitable for our biosensors, but it may be useful for many other applications. During Phase I ethanol was used to demonstrate that the general techniques proposed could be successfully applied to an FAD dependent and unstable enzyme. We demonstrated the ability to drive the expression of all the alcohol oxidase constructs into inclusion bodies, but folding the protein from the inclusion bodies has not yet been shown. In Phase II, we instead focused on evolving a cortisol oxidase. Cortisol is both a very important biomarker, and the particular evolution being proposed could lead to an entire class of oxidases that exhibit extreme thermal stability while remaining active and selective. While we still believe that this approach is viable, in this case, we were not able to produce an oxidase that showed significantly more activity toward cortisol then the starting wild type enzyme. However, as a result of this effort, we have produced a cholesterol oxidase that is stable in both temperature and detergents. The overall plan for this development effort is shown in Figure 1. Biosensors were used both as an end product, and as a mechanism to evaluate the efficacy of the immobilized enzymes for the purposes of generalized diagnostic measurements.

Figure 1 Project Overview



3 METHODS, ASSUMPTIONS, AND PROCEDURES

3.1 Evolution of Cortisol Oxidase

In this Task our goal was to develop a new enzyme for diagnostic device applications: Cortisol Oxidase. Guided by our past efforts and the literature, we used modern molecular biology in an attempt to engineer an existing oxidase, cholesterol oxidase into an enzyme that is specific for cortisol.

Through regular, ongoing testing we continually evaluated the latest generations of the evolved cortisol enzyme to ensure that changes to the enzyme did not result in unintended characteristics that preclude its use in device fabrication. Identifying potentially adverse mutations as early as possible in the development cycle allowed us to further refine the selection process.

3.1.a Repurposing of Cholesterol Oxidase as a Cortisol Oxidase

The only oxidase enzymes that act on a steroid backbone are the cholesterol oxidase family. Cholesterol, another important human response molecule, has some structural similarity to cortisol (Figure 2), and as such, offered the most rational starting point for the engineering of a cortisol oxidase. It has been well established that the 3' hydroxyl moiety on the A-ring (circled) of cholesterol is the site of oxidation. The analogous site on cortisol is the hydroxyl moiety at the 11' C-ring (circled). Although the similarity between these two steroidal compounds may seem distant, we believed that the structural homology was close enough to provide a useful starting point for the evolution of a cortisol oxidase. Pragmatically, the evolution of a cortisol oxidase from a cholesterol oxidase was the best and perhaps only chance of success.

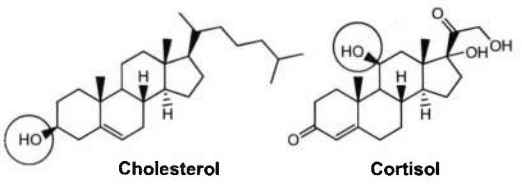


Figure 2 Molecular structures of cholesterol (left) and cortisol (right) revealing the steroidal backbone common to both species. The sites of oxidation are circled.

Many different cholesterol oxidases have been described in the literature, and that from *Streptomyces sp.* is the best characterized.[11, 23-39] Multiple x-ray crystal structures of this protein exist, including co-crystal structures with bound substrate analogs.[24,28,33] A non-stabilized, essentially unformulated version of this enzyme is commercially available. We showed that this enzyme has some basal activity against cortisol, albeit at a rate that is 10^6 fold reduced from cholesterol. We also showed that the lactate-like side chain in cortisol is not a substrate for lactate oxidase. This precluded the use of lactate oxidase as a starting point for the development of a cortisol sensor. As part of this study, we also performed a detailed analysis of the existing cholesterol crystal structures, which included hand docking cortisol into the cholesterol active site. We believe that the structural basis for this decrease in activity is the orientation of cortisol in the active site and the proximity of the cortisol hydroxyl to the riboflavin ring (Figure 3).

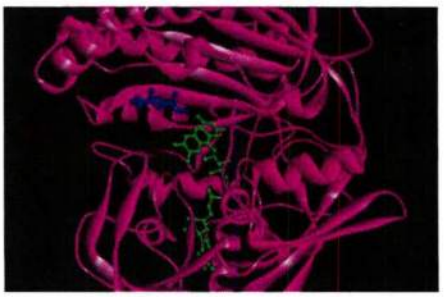


Figure 3 Model of cortisol binding into the active site of *Chromobacterium sp. DS-1* cholesterol oxidase. Blue = cortisol backbone; Green = FAD; Magenta = Cholesterol Oxidase.

A newly discovered second form of cholesterol oxidase from *Chromobacterium sp. DS-1* was also investigated. This enzyme is significantly more thermally stable, more resistant to denaturation by organic solvents and more resistant to denaturation by detergents than the *Streptomyces sp.* enzyme.[25-27] The *Chromobacterium* cholesterol oxidase also appears to better maintain co-factor residency and consequently activity. This species has been cloned and isolated, and a single crystal structure (without bound substrate) is available (RCSB accession code: 3JS8). Given the overall stability profile of the *Chromobacterium* enzyme, we believed that this species was the ideal starting point for the evolution of cortisol oxidase with enhanced thermal stability and co-factor stability profiles. Furthermore, we believed that the binding affinity of the native enzyme is rate limiting for cortisol activity until the K_d is reduced by at least 10^3 relative to cholesterol. That is, the enzyme must demonstrate a higher affinity for cortisol. Finally, we hypothesized that by reducing the K_d for cholesterol, we would observe a concomitant increase in k_{cat} for cortisol in a transformed cholesterol oxidase.

3.1.b Cloning, expression and purification of cholesterol oxidase from *Chromobacterium sp. DS-1*

The gene for cholesterol oxidase from *Chromobacterium sp. DS-1* was cloned, and the protein product expressed and purified according to literature procedures.[25-27] This enzyme was assessed for activity against cortisol.

A series of vectors were prepared based on pTBSG[40] which contain a nucleotide sequence encoding a T7 promoter followed by a His_6 tag then by genes encoding either GST, MBP, SUMO or GB1 fusion proteins, each with a flanking KpnI and SpeI restriction site, followed by a TEV cleavage site. An open reading frame was cloned using the SspI independent cloning (LIC) site into any fusion vectors. This arrangement is shown in **Error! Reference source not found.**. In this Subtask, the genes encoding the wildtype *Chromobacterium sp.* DS-1 cholesterol oxidase enzyme were synthesized commercially (*Genscript, Inc.*) and cloned as described.[40] The fusion constructs were transformed into *E.coli* XL1 blue cells for plasmid amplification and then into *E.coli* BL21 cells for protein over-expression.[41]

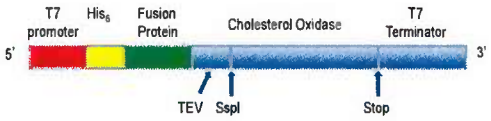


Figure 4 Representation of the pTBSG vector.

Small-scale protein expression in *E. coli* was used as an initial screen to quickly evaluate the effects of the different fusion proteins on the stability of the recombinant *Chromobacterium sp.* cholesterol oxidase with respect to enhanced co-factor residency (retention of bound flavin nucleotide) or thermal stability. The stoichiometry of the bound flavin was assessed spectrophotometrically in multiwall plates by determining the ratio of absorbance at 454 nm to that at 280 nm.[42] The fusion protein and 6His tag were removed, when required, by adding TEV protease.[43]

3.1.c Development of a Designed Cortisol Selection Screen

We successfully implemented random screens to screen large libraries of oxidase mutants for enhanced properties (For example, see Figure 5). This approach is random, and therefore less optimal than functional or semi-functional directed evolution paradigms. However, our studies have shown that while random, this approach is still a viable method for searching for oxidase

mutants with altered properties. This screening paradigm is a backup to the designed or semi-functional cortisol screen described below.

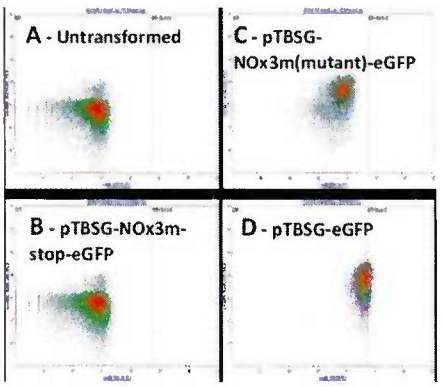


Figure 5 Results of a FACS-based random selection screen for enhanced nicotine oxidase enzymes – a screen that could be used for the identification of new cortisol oxidase enzymes.

The semi-functional screen leverages our observation that cholesterol oxidase has some basal level of activity towards cortisol. A selection procedure was designed in which (i) cholesterol is substituted for cortisol as a carbon source, (ii) the *Chromobacterium sp. DS-1* cholesterol oxidase gene was randomized, and (iii) the peroxide produced by the action of the cholesterol oxidase was monitored directly on the growth medium for the organisms with the randomized *Chromobacterium sp. DS-1* cholesterol oxidase gene inserted into the host cells.

Directed evolution was used to modify the catalytic properties of *Chromobacterium sp.* cholesterol oxidase to make the enzyme more suitable for cortisol oxidation. Directed evolution is a rapidly emerging approach exhibiting exceptional promise for engineering enzymes with altered or new capabilities,[44] and uses a variety of functional screens for selection.

Our approach was to start with the preparation of mutant gene libraries by random mutagenesis. This library was then expressed in *E. coli*, and selected for a desired property – in this instance the ability to oxidize cortisol and produce H_2O_2 as a by-product which could be used as part of a diagnostic device monitoring scheme. A colony-staining method based on the principle of Allain's method was used for the screening of cortisol oxidase.[38, 39] Protein libraries were screened using growth rate and cortisol concentration as selection parameters to identify positive mutations. This directed evolution approach could be used for multiple rounds of selection as the cortisol oxidation properties were optimized.

In this approach, an Error-Prone PCR was employed to randomly introduce amino acid substitutions into the cholesterol oxidase gene and the resulting mutant libraries were screened for improved specificity, stability and efficiency (reviewed in Roodveldt et al.[45]). The mutation rate could be adjusted by varying the $MnCl_2$ concentration between 0 and 125 µM. After determining the $MnCl_2$ concentration needed to achieve the desired mutation rate, a mutant library of ~1,300 active cholesterol/cortisol variants was obtained. The PCR products were inserted into the pTBGS vector and transformed into *E. coli* expression strain BI21(DE3).

The cultures of expression strains were grown on an agar M9 minimal medium supplemented with cortisol as sole carbon source. Filter papers dipped into a solution containing color developing reagents were placed on colonies. Cortisol oxidase activity of the test colonies was indicated by a red color due to the formation of quinoneimine dye.[25, 26] Doukyu's group previously discovered that *E. coli* carrying a plasmid containing the gene of cholesterol oxidase formed turbid halos around their colonies on an agar medium containing cholesterol because of the low solubility of the oxidation product.[27] We expected a similar solubility issue with the formation of the cortisone (by product of cortisol oxidation). Therefore, we used this turbid halo formation with the agar medium as our primary screen, but solubility was an on-going issue. Individual clones from the selected sub-population were grown separately and examined by eye. The genes from

colonies giving rise to the cortisol oxidase activity were replicated for sequencing, expression, purification, and activity tests.

3.1.d Use of a random, FACS-based mutant selection protocol for a cortisol oxidase

As described in the Results, directed evolution did not yield the desired results for this effort. Random mutagenesis and screening was also used to modify the catalytic properties of cholesterol oxidase for cortisol processing.[44] We have used this screen successfully for other oxidase enzymes (Figure 5 and Figure 6).

The native *Chromobacterium sp.* cholesterol oxidase gene (*Cb*-CHO) was fused C-terminal to the enhanced green fluorescent protein (eGFP) as a quantitative reporter,[46-49] which was cleaved off by TEV co-expressed during selection.[50] The resulting construct was amplified by Error-Prone PCR (EPP) following the method of Fromant[51] to randomly introduce amino acid substitutions into the *Cb*-CHO-eGFP gene. The resulting libraries were screened for improved activity (methods reviewed in Roodveldt[45]). As eGFP fluorescence is readily measured, and as there is a 1:1 molar correlation between the target protein and GFP, we normalized the growth/expression variation among the clones, facilitating primary screening of error-prone PCR (EPP) variants. These libraries are also amenable to high throughput functional screening to further improve selection.

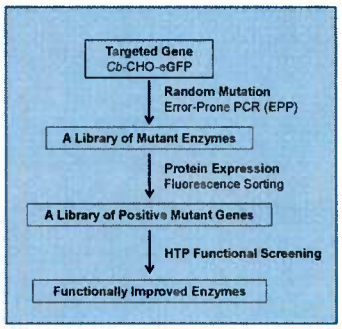


Figure 6 Flowchart of the proposed random mutant selection screening protocol

The mutation rate could be adjusted by employing different $MnCl_2$ concentrations (from 0 to 125 mM) to achieve mutant libraries of ~1,300 active cortisol oxidase variants per library. The libraries were screened via eGFP fluorescence FACS sorting. The PCR products was inserted into the high throughput cloning vector pTBGS that will add an eGFP gene at the C-terminal of the fusion protein. The plasmids were transformed directly into *E. coli* expression strain Bl21(DE3)pRK603 that produced TEV protease to cleave eGFP from the fusion protein.[50] The cultures of expression strains were diluted in PBS to a density at which individual cells could be sorted by flow cytometry (BD Biosciences FACSVantage SE). In the first round, cells from the top 1% fluorescent cells were sorted directly into growth medium. Individual clones from the selected sub-population were grown separately and examined by measuring whole cell fluorescence intensity. The genes giving rise to the brightest fluorescing cells were replicated for sequencing, expression, purification, and activity tests. The plasmid libraries of positive mutant *Cb*-CHO genes from eGFP screening were expressed in *E. coli* cells that are naturally permeable to cortisol in a 96-well format. Cells were grown in LB first and then switched to M9 medium supplemented with cortisol and grown for an additional 60 minutes in the presence of IPTG. The cells were pelleted and the medium absorbance measured to screen for the disappearance of cortisol (absorbance change at 231 nm) using a microtiter plate reader. The mutants that most efficiently removed cortisol from the medium were replicated and analyzed. The entire screen took place in a 96-well format.

3.2 Large Scale Enzyme Production and Formulation

This Task was designed to optimize the large-scale expression, purification and formulation of enzymes to maintain the improvement of co-factor residency, optimize shelf-life stability and provide materials needed for the testing of the enzymes when integrated into a diagnostic device (Section 3.3).

The success of our Phase I studies demonstrated that for some oxidases, addition of an N-terminal fusion protein improved the enzyme's co-factor residency while maintaining the catalytic fidelity of the system, and also improved the overall thermal stability of the enzyme. Part of this Task was to optimize the formulation of the Phase I enzymes to maximize the shelf-stability of these species.

3.2.a Large Scale Enzyme Production Our preliminary observations identified two potential pathways to optimize the production of active oxidases for device development. The first was maximizing the expression of soluble enzyme. The advantage to this approach was that the enzymes were already in their native, active conformations. However, the yields of soluble enzyme vary widely from one preparation to another and from one enzyme to another. When low yields are obtained, multi-step purification is needed to obtain near-homogeneous enzyme preparations. This can be costly and time consuming. Alternatively, proteins obtained in inclusion bodies are reliably produced in very high yields and are readily purified, promising quantitative production of gram quantities of enzymes. Inclusion bodies can be stockpiled and stored indefinitely. The challenge was to develop efficient and robust folding methods to recover the active enzyme forms from inclusion bodies, suitable for commercial use. To ensure successful oxidase production on a scale suitable for prototype device preparation, both pathways were explored.

All protein isolation experiments were done on 1 L cultures. For many oxidases, 1 L cultures can provide 100 mgs (or more) of purified protein. Yield was determined from the amount of final, purified protein (>95% pure) per liter. Protein purification took place using standard conditions (Ni column, desalting, etc.).

Each of the evolved enzymes underwent expression testing at 10, 17, 25 and 37 °C and the optimal temperature was used for preparation of each 1 L culture. For oxidases, this temperature survey is important for identifying optimal conditions when scaling to 1 L and beyond, and it is also critical for identifying the optimal conditions for soluble expression. We also drove the expression of the enzyme into inclusion bodies at 37 °C and refolded the enzyme *ex vivo*. While there is an obvious preference for the production of only soluble protein, on a large scale there are advantages to producing the oxidase as an inclusion body.

3.2.b Increasing the yield of soluble proteins In some instances, the soluble expression of an oxidase enzyme could be further enhanced by increasing the overall solubility of the enzyme. In a recent study of an engineered recombinant nicotine oxidase, we introduced surface mutations at five separate positions on the enzyme surface that were predicted by the *program I-Mutant2.0 (10) to* enhance enzyme stability. *I-Mutant2.0, w*hen trained, tested, and cross-validated against a known crystal structure, correctly predicts which single mutations can stabilize a protein with 80% accuracy. For example, in the case of nicotine oxidase, a combination of two of the five surface mutations resulted in a four to six-fold increase in recovery of the MBP-nicotine oxidase mutant over that of the wild type MBP-nicotine oxidase in soluble form. A similar approach was used in this project with the recombinant MBP-tagged oxidases following application of the *I-Mutant 2.0* algorithm to identify mutation sites that were likely to enhance solubility possibly with the added benefit of enhanced thermal stability.

3.2.c Folding from inclusion bodies An alternative to increasing the expression of soluble protein is to drive the protein expression to produce inclusion bodies. The folding process is then completed *ex vivo*. Our Phase I folding study resulted in the recovery of small amounts of LacOX and MBP-LacOX, which represented a good starting point for this effort. Two approaches were

used to build on this result to develop a robust methodology for oxidase folding. The first approach was more traditional involving a systematic study of folding conditions, including varying the concentration of urea/guanidine-HCl, the protein concentration, the temperature, substrate and cofactors and osmolyte concentration and chaotropes for retention of solubility. This approach was used to obtain an active recombinant aryl-alcohol oxidase, one of the very few published oxidase folding studies.[52] We have also used this approach with several proteins to successfully obtain more than 50% yields of correctly folded and stable proteins.[41, 53-57] The second approach involved a new methodology involving the use of molecular chaperone proteins in the presence of high concentrations of osmolytes.[58, 59] This method has the potential to be adapted to large-scale protein folding in a manner that may ultimately lead to a platform-type technology. This was tried, when needed, to enhance the refolding of inclusion body preparations of the target enzymes.

3.2.d Enzyme Lyophilization Optimization In general, when fabricating a diagnostic device, it is preferable to use enzyme that is in a solid (lyophilized) form. For oxidases, the lyophilization process often requires the addition of a stabilizing agent. This agent could be a small glycol, or a simple mono- or di-saccharide. In our experience, once the appropriate stabilizing agent for a particular wild-type protein is identified, this agent is often suitable for use with mutant forms of the protein.

For each enzyme, we surveyed the common lyo-protectants, such as sucrose, trehalose and 2-methyl-2,4-pentanediol, at a range of concentrations (as needed) for the ability to stabilize the lyophilized form of the evolved proteins. We also compared the enzymes formed as a suspension in glycerol and sucrose. For each preparation, we spectrophotometrically assessed the enzyme activity before and after lyophilization,[60]. The best lyo-protectant was that which retains the highest specific activity of the enzyme, which by definition, is the protectant that optimizes co-factor residency.

3.2.e Enzyme Stability Assessment The stability of the lyophilized enzymes was assessed for optimal shelf-life when stored at three different temperatures: 25°C, 0°C and -80°C. We assessed each preparation spectrophotometrically[60] for the enzyme's activity as a function of time and temperature.

3.3 In Vitro and In Vivo Validation

The enzymes resulting from 3.1 and 3.2 were tested for their performance when integrated into a diagnostic device. Specifically, we used biosensors to characterize the *in vitro* and *in vivo* performance of the enzymes produced (Figure 7). Just because an enzyme is available does not mean that the enzyme will function correctly when immobilized as part of a biorecognition element.

3.3.a Biosensor Fabrication The key limitation in the development of a new biosensor is the availability of a stable oxidase enzyme that is analyte specific. We produced new biosensors for lactate, and histamine from the lyophilized enzyme products formulated in 3.2.

Biosensor construction has been described previously.[61, 62] Briefly, a Teflon-coated Pt–Ir wire forms the working electrode (uncoated diameter: 180 µm, coated diameter: 240 µm). The reference electrode is formed by wrapping a silver wire (diameter: 50 µm) concentrically around the Pt–Ir wire. The sensing cavity, which houses both the biological recognition element (an

oxidase enzyme) and the transduction element (exposed platinum-iridium surface), is formed at the distal end of the working electrode by removing a 1 mm section of the Teflon coating.

A selective membrane is used to protect the electrode surface from endogenous electroactive species (i.e. ascorbic acid). This membrane may be formed by traditional coating methods or electropolymerization of a phenol or pyrrole derivative. The exact nature of the film was dependent upon the specific needs of the enzyme/analyte interaction. This was determined in a routine manner during biosensor fabrication. In addition to the passive selective membrane, ascorbate oxidase may be co-immobilized along with the biological recognition element as a secondary method of ascorbate rejection. Ascorbate oxidase, unlike many other oxidase enzymes, produces H_2O as one of its enzyme products instead of H_2O_2. This allows for the direct removal of ascorbate (one of the most prevalent interfering species) from the sensing cavity, but it also increases the amount of oxygen required.

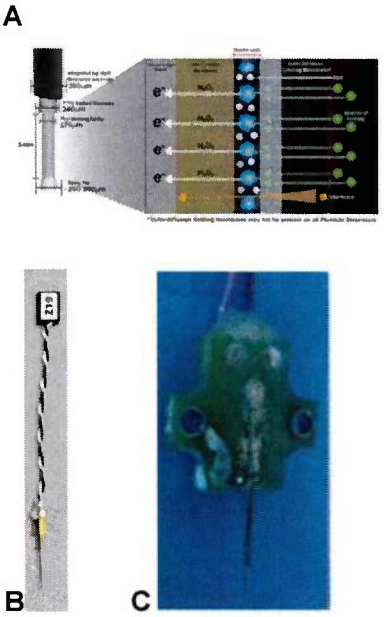


Figure 7 (A) Generalized schematic diagram of a commercial Pinnacle biosensor. (B) A commercial Pinnacle biosensor (Model 7004) for *in vivo* monitoring in the brain of rodents. (C) A prototype Pinnacle biosensor for subcutaneous analyte monitoring.

The overall sensitivity of the final biosensor is dependent upon the activity and stability of the immobilized enzyme. Immobilization of the enzyme (the biological recognition element) to the sensor's transduction element (in this case a platinum-iridium electrode) can be accomplished through a variety of means. Taking into account the needs of a commercial manufacturing process, Pinnacle has found the classical method of enzyme immobilization by glutaraldehyde and bovine serum albumin (BSA) cross-linking to be not only the most robust and reliable method, but also the most conservative in terms of enzyme consumption per electrode. In an optimized electrode, BSA serves as a protein backbone upon which the active oxidase enzyme is immobilized by glutaraldehyde crosslinking. This backbone provides a substructure for the covalent bonding of the oxidase enzyme. Without the BSA protein, the oxidase enzyme would be left to crosslink with other oxidase enzyme units. Too much of this type of 'self' crosslinking can severely diminish the immobilized activity of the enzyme. Therefore, it is extremely important to tailor the amount of BSA in the crosslinking process to optimize the immobilized activity of the enzyme.

The actual makeup of the immobilization matrix was determined to optimize the activity of each immobilized enzyme. The optimal makeup is dependent upon the characteristics of the enzyme. If required, a diffusion limiting membrane was added. This is necessary when the physiologically relevant concentration range of the analyte exceeds that of enzyme's K_M.

3.3.b In Vitro Testing The *in vitro* performance of each enzyme when integrated into a biosensor (lactate, histamine, and cortisol) was evaluated. For the purposes of this study, the biosensors were the diagnostic device used to evaluate enzyme stability, shelf-life and performance of the species from 3.1 and 3.2. These tests are direct measures of an enzyme's performance characteristics when integrated into a diagnostic device (Figure 7).

3.3.c Sensitivity Testing Each enzyme's sensitivity when integrated into a diagnostic device was tested. Analyte was added to the system in stepwise fashion to determine the limit of detection, linearity and the overall sensitivity of the biosensor. All electrodes made for shelf-life testing and to support *in vivo* tasks were tested in this manner.

3.3.d Shelf-Life Testing The shelf-life stability of each enzyme when integrated into a diagnostic device was tested. To determine shelf-life and optimal storage conditions for each formulation, three cohorts of thirty biosensors were manufactured and all were tested for analyte sensitivity on Day 0. Cohort 1 was retested after 7 days, Cohort 2 after 14 days and Cohort 3 after 21 days. This separation was to ensure that each biosensor was only tested twice, the initial test and the life-time test. The biosensors in each cohort were further divided into five groups containing six biosensors to test different storage conditions. Storage conditions were: 0 °C, 4 °C – dry, 4 °C in buffer, 25 °C dry, and 25 °C in buffer. Yield and degradation profiles characterizing the shelf-life expectancy of all groups for each biosensor type were assessed.

3.3.e In Vivo Testing The *in vivo* performance of each enzyme when integrated into a biosensor was evaluated. In all cases, Sprague-Dawley rats were used for animal testing.

All animal work completed as part of this contract was performed within the University of Kansas Animal Care Unit (Animal Welfare Assurance #: A3339-01) and Army approval (AMRDEC – 2946). No animal work was conducted until all necessary approvals (University of Kansas IACUC and US Army ACURO) were obtained. The KU IACUC approval was granted on April 19, 2013 under the designation Animal Use Statement #215-05. The approved animal use statement was then forwarded to the US Army Animal Care and Use Review Office (ACURO) along with the completed ACURO Animal Use Appendix. ACURO approval was obtained on June 17, 2013. Due to a change of the Principal Investigator from Dr. Peter Petillo to Dr. David Johnson, the KU Animal Use Statement number was changed from 215-05 to 228-04 (IACUC approval date 11/24/15).

3.3.e.1 CNS Testing

Anesthetized Animal Testing: The *in vivo* limit of detection (LOD) for each enzyme in a biosensor was determined. The LOD is dependent upon the performance of the enzyme acting as the biosensor's recognition element. This test allowed us to directly quantify the performance of the enzyme in an *in vivo* environment.

Animals were maintained under anesthesia throughout the experiment. The biosensor was stereotaxically implanted in close proximity (within 1 mm) to an infusion port (guide cannula/needle) in the striatum (A/P+1.0, M/L+2.5, D-5.5) or cortex (A/P+2.75, M/L+0.5, D-1.0).

Biosensor sensitivity was evaluated *in vivo* by multiple, increasing local administrations of analyte through the infusion port. The limit of detection (LOD) was estimated based on the dose-response curves and the post-calibration of the sensors was determined. While these studies did not rely on the physiologic release of the analyte, they provided important information regarding the performance and functionality of the stabilized enzymes.

Freely Moving Rat: The performance of the enzyme in an *in vivo* environment responding to physiologic release of the analyte of interest and the *in vivo* lifetime of the enzyme were assessed. Sprague Dawley rats were stereotaxically implanted with guide cannulas aimed at the appropriate brain region. A head mounted enclosure was situated around the guide cannulas and secured to

the skull with bone screws and dental cement. Animals were allowed a recovery period of at least 5 days following surgery. Following the recovery period, biosensors were lowered into the implanted guide cannula and a wireless potentiostat was added to the headmounted enclosure (Pinnacle Model 8172 wireless potentiostat). Animals were returned to their home cage for continuous biosensor recording. The specific experimental paradigms for each analyte are described in 3.3.a.

3.3.e.2 Subcutaneous Testing The biosensors were tested in a subcutaneous environment by implanting Pinnacle's subcutaneous sensors (Figure 7). These devices were designed as a platform for robust, rugged performance while implanted and include a wireless Bluetooth transmitter. The implants were sterilized with ethylene oxide and installed aseptically while the animal was anesthetized (isoflurane). The implant was mounted on the animal's back and protected by medical tape and an infusion jacket.

Once the implant was in place, the biosensor was allowed to stabilize *in vivo* and then a bolus of the analyte was injected into the local tissue. We recognize that the diffusion of a bolus from a subcutaneous injection is imperfect, and that the biosensor was not exposed to the full concentration of the bolus. Once the resulting signal returned to baseline, the implant was secured by medical tape and the infusion jacket. The rats were returned to their home cage. Following an interval of up to four days, the animal was anesthetized again; the implant was exposed, and a bolus of the analyte was injected to the tissue surrounding the implant. The animal remained anesthetized until the signal returned to baseline when the implant was re-secured and the animal returned its home cage. This cycle was repeated until no change in signal was observed upon dosing.

3.3.f Lactate Oxidase Testing

The lactate biosensors underwent both CNS and subcutaneous testing.

In the CNS surgery, guide cannulas aimed at the pre-frontal cortex were implanted. Lactate was injected in boluses in the range of 100 to 1000 µM. We previously showed that lactate concentration changes with sleep state in mice.[63] Lactate increases with waking and decreases with transition to sleep state. We used this relationship to validate the response of the lactate biosensor *in vivo*.

Following the surgery and recovery period described above, lactate biosensors were lowered into place through the guide cannula and connected to a wireless potentiostat mounted in the head-mounted enclosure. The animals were returned to their home cage and recording began immediately. Recording continued until no change in signal was noted as the animal transitioned between wake and sleep states.

Subcutaneous biosensors were implanted as described in 3.3.e.2. Bolus dosing of lactate was carried out by delivering a 100 µM lactate bolus dose to the area surrounding the implanted biosensors. Dosing was repeated every four days until no change in signal was observed.

3.3.g Histamine Oxidase Testing

The histamine biosensors underwent both CNS and subcutaneous testing.

A guide cannula aimed at the pre-frontal cortex was implanted. Histamine was injected in increasing boluses in the range of 0.01 to 20 µM. Histamine has been shown to increase upon injection (IP) of the H3 receptor antagonist thioperamide [64, 65] The histamine biosensor was tested using thioperamide to elicit a change in histamine concentration. Sprague Dawley rats were implanted with guide cannula as described above. However, in this case, cannulae were aimed at the basolateral amygdala or hippocampus. After recovery, rats were injected with vehicle, and then followed one hour later by thioperamide (5-25 mg/kg IP). Dosing was repeated daily until there is no change in signal upon dosing. Results were compared to previous studies.

Subcutaneous biosensors were implanted as described above. Bolus dosing of histamine was carried out by injecting 0.1 to 1 mL of 1% histamine. Dosing was repeated every four days until no change in signal is observed.

3.3.h Cortisol Oxidase Testing

As discussed in the Results section, we have not been able to produce a cortisol oxidase suitable for this type of biosensor, therefore no *in vivo* testing occurred.

4 RESULTS AND DISCUSSION

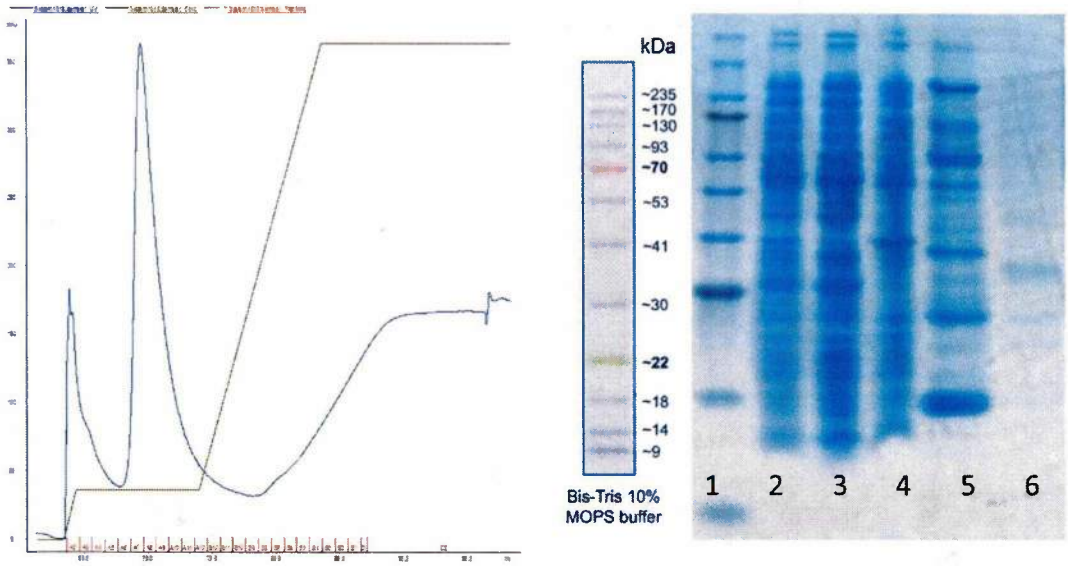
4.1 Cortisol Oxidase Evolution

4.1.a Cholesterol oxidase from *Chromobacterium and Brevibacterium*

The DNA encoding the genes for cholesterol oxidases from *Chromobacterium sp. DS-1* (RCSB ascension 3JS8) and *Brevibacterium sterolicum* (RCSB ascension 1COY) as well as the primers needed for the cloning were examined. We made the decision to look at both variants of cholesterol oxidase to increase our chances of success. The DNA was cloned into a pTBSGvector containing a nucleotide sequence encoding a T7 promoter followed by a His6 tag (**Error! Reference source not found.**). This allowed the expression of the two genes as a starting point. Therefore, the genes encoding either GST, MBP, SUMO or GB1 fusion proteins, each with a flanking KpnI and SpeI restriction site, could be readily inserted. The constructs were transformed into *E.coli* XL1 blue cells for plasmid amplification and then into *E.coli* BL21 cells for protein over-expression.

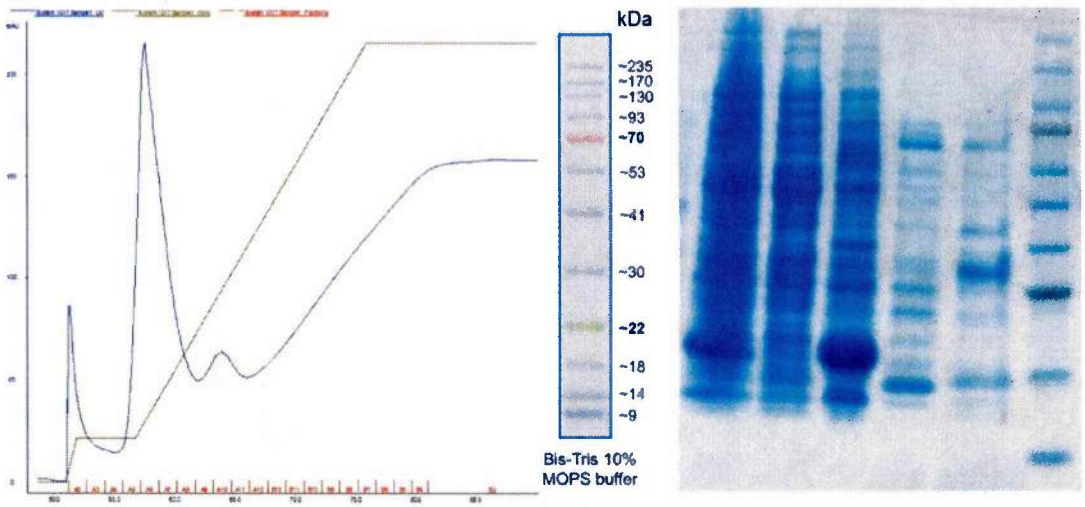
For the 1COY construct, the cloning was uneventful and sequencing proved that we had the correct DNA sequence. We subsequently examined conditions that optimized soluble expression (at 17 and 37 °C) as well as conditions designed to drive the protein into inclusion bodies. None of the conditions examined were successful in producing more than microgram quantities of protein (based on inspection of gels). These data were all collected on the wild-type protein in the absence of a MBP or SUMO tag.

We continued to look for conditions that optimized soluble expression (at 17 and 37 °C) as well as conditions designed to drive the protein into inclusion bodies (Figure 8 and Figure 9). Expression and purification at 17 °C yielded small quantities of soluble product (Figure 8, lanes 2-4) on the order of 10-20 ug/liter of culture. Expression and purification at 37 °C yielded large quantities of insoluble, cleaved protein products (Figure 9, lane 3).



Lane 1: 1COY @ 17 °C whole cell lysate
Lane 2: 1COY @ 17 °C soluble fraction
Lane 3: 1COY @ 17 °C insoluble fraction
Lane 4: 1COY @ 17 °C A7peak
Lane 5: 1COY @ 17 °C B7 peak
Lane 6: GoldBio BLUEStain ladder

Figure 8 Purification Elution Profile from AKTAXpress of 1COY Construct Expressed at 17 ^{0}C



Lane 1: 1COY @ 37 °C whole cell lysate
Lane 2: 1COY @ 37 °C soluble fraction
Lane 3: 1COY @ 37 °C insoluble fraction
Lane 4: 1COY @ 37 °C A6 peak
Lane 5: 1COY @ 37 °C A10 peak
Lane 6: GoldBio BLUEStain ladder

Figure 9 Purification Elution Profile from AKTAXpress of 1COY Construct Expressed at 37 ^{0}C

Once the 1COY gene was verified, the fusion epitope was inserted into the construct. Based on historical precedence, both MBP and SUMO tags were added to the gene. Both constructs (MBP-COY1 and SUMO-COY1) were expressed at 37° C and driven in inclusion bodies. After expression, the cells were stored in a 20 mM Phosphate pH 7.4 @ 4° C + 300 mM NaCl buffer at -80° C. Protein from both constructs were recovered and assayed for total protein concentration (Table 1).

Table 1 Protein from Cholesterol Oxidase constructs

	mg/mL	Total protein from 1L
MBP-COY1	14.1	199
SUMO-COY1	22.2	177

As with the other oxidase constructs that we investigated, the recovery of inclusion bodies is exceptional (Figure 10). Initial folding studies of these two constructs used the lactate oxidase folding conditions. While a reasonable starting place, the process of identifying folding conditions is highly empirical and a number of iterations were required to produce active protein. Given the amount of protein that is produced by the inclusion bodies, this route still represents the preferable method for the large-scale production of cholesterol oxidase.

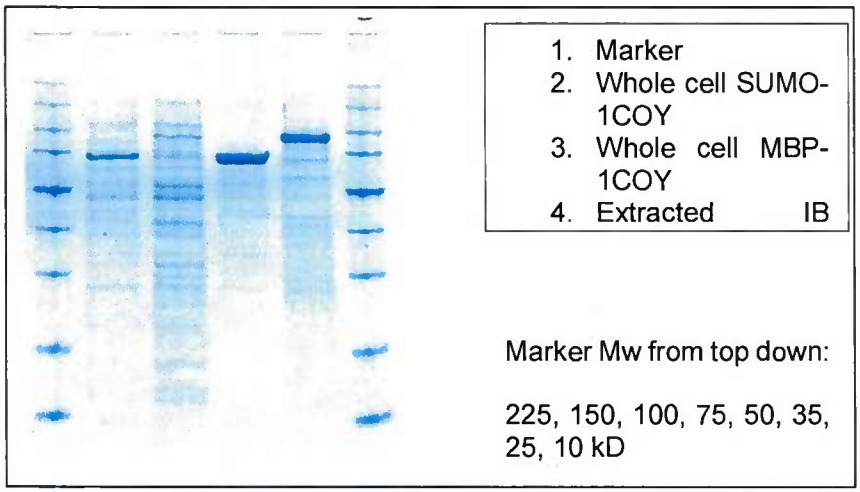


Figure 10 Expression profiles of SUMO-1COY cholesterol oxidase constructs for inclusion body preparations

In summary, we successfully cloned, expressed and purified small amounts of six different cholesterol oxidase constructs: wt-COX1, SUMO-COX1, MBP-COX1, wt-COX2, SUMO-COX2 and MBP-COX2 (Table 2). COX1 refers to the gene product from *Brevibacterium sterolicum* and is associated with the 1COY crystal structure. COX2 refers to the gene product from *Chromobacterium sp. DS-1* and is associated with the 3JS8 crystal structure. Excellent expression was observed for all of the constructs (Figure 11) with the exception of wild-type COX1. With the exception of wt-COX1, the Kcat's of all the constructs are excellent. Importantly, when adjusted for the FAD content, the data show that the presence of the fusion protein does not dramatically compromise the catalytic activity of the enzymes, and in the case of MBP-COX2,

kcat is somewhat improved. The important observations from these data are (1) that presence of the fusion protein does not cause the catalytic nature of the enzyme and (2) the presence of the fusion protein does improve co-factor residency. For the purposes of comparison, Table 2 also includes data for commercial cholesterol oxidase from *Rhodococcus sp.*

Table 2 Summary of COX1 and COX2 *soluble* expression results. All proteins were expressed @ 22 °C for 16 hours and purified under standard conditions. Proteins (3ug protein) were assayed using Amplex Red and 200 uM cholesterol dissolved in 0.5% (v/v) triton X-100.

	Wt-COX1	SUMO-COX1	MBP-COX1	Wt-COX2	SUMO-COX2	MBP-COX2	Commercial COX
Yield (mg / 1L culture)	4.1	22.0	52.4	56.7	74.5	39.7	NA
Off Column FAD/Protein	~0.01	0.3	0.4	0.6	0.7	0.7	0.3
unadjusted SA (U/mg)	<0.01	1.7	1.5	5.5	5.3	3.8	2.4
SA adjusted for FAD ratio (U/mg)	0.02	6.8	3.9	10.0	7.2	5.6	9.8
Unadjusted Kcat /sec	<0.01	2.2	2.6	5.2	6.7	6.5	1.8
Kcat adjusted for FAD ratio/sec	0.2	8.8	6.7	9.4	9.0	9.6	7.3

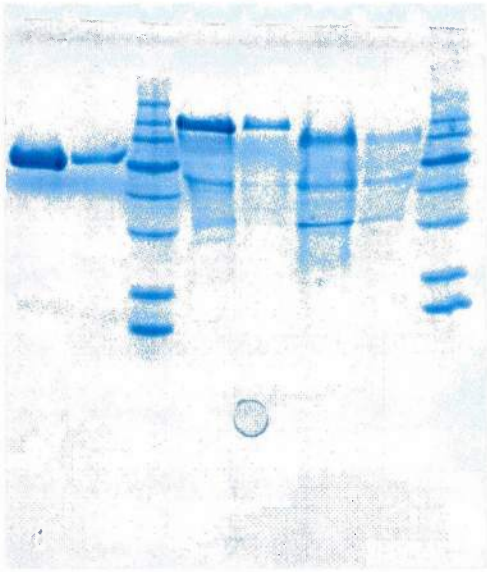


Figure 11 Summary of expression testing of cholesterol oxidase constructs. Wt-COX1 and MBP-COX1 were expressed at 22 °C for 16 hours. Marker molecular weight from top down: 225, 150, 100, 75, 50, 35, 25, 10 kD. Lanes from left to right: 1) COM-COX, 5ug; 2) COM-COX, 1ug; 3) MW Marker; 4) MBP-COX1, soluble, 5ug; 5) MBP-COX1, soluble , 1ug;6) COX1, soluble, 5ug; 7) COX1, soluble, 1ug; 8) Marker

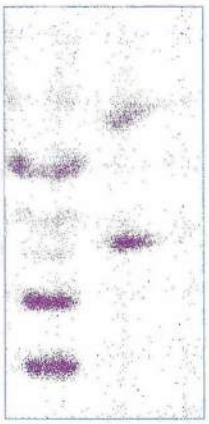
4.1.b Cortisol Selection Screen

The strategy we used was to randomly mutagenize the COX2 gene (that corresponded to the 3JS8 crystal structure) via PCR, and then transform the randomized DNA into cells suitable for growth on minimal media (M9) plates that were supplemented with cortisol. Colonies that could grow on the M9 plates, by definition, were able to use cortisol as a carbon source.

4.1.b.1 Plasmid transformation basic procedure

Plasmid pTBSG-COX2 was transformed into DH5α competent cells. DH5α cells (50 uL) were thawed on ice, and then 5 uL pTBSG-COX2 (78.8 ng/uL) was added to the cells. DH5α cells with plasmid were incubated on ice for 30 minutes and then heat shocked at 42 °C for 45 seconds. After incubation on ice for another 5 minutes, 950 uL SOC media was added to the cells. The cells were incubated at 37 °C for 1 hour with vigorous shaking (250 rpm). The DH5α cells with plasmid were plated on LB agar plate with 100 ug/uL ampicillin and incubated at 37 °C overnight.

Three colonies from pTBSG-COX2 DH5α plate were picked. Each colony was added into 10mL LB media inoculated with 100 ug/uL ampicillin and incubated at 37°C overnight with vigorous shaking (250 rpm). The pTBSG-COX2 DH5α plasmids were extracted from each culture using EZNA miniprep kit.



The randomly mutated pTBSG-COX2 was supplemented with 0.5uL DpnI and digested at 37 °C for 2 hours, then the digested product was column-purified using EZNA Cycle Pure Kit. The purified mutagenic COX2 was T4-treated with the following reaction. The sample was incubated at 22°C for 30 minutes and heated at 75 °C for 20 minutes. 1uL T4-treated mutagenic COX2 was supplemented with 0.5uL pTBSG vector (T4-treated, PCR opened) and 0.5uL EDTA and incubated at 22 °C for 5 minutes.

Figure 12 Agarose gel of PCR insert of randomized pTBSG-COX2 DNA. Lane 1 is the MW marker and Lane 2 is the amplified insert and plasmid.

4.1.b.2 Preparation of M9 plates with cortisol substituted for glucose as the sole carbon source

80 mL of double distilled water, 1.5 g of agar and 0.1 g NH_4Cl was added to a flask. This mixture was then autoclave sterilized. Subsequently, 1 mmol of hydrocortisone (0.36 g) was dissolved into 30 mL ethanol and filter-sterilized. After the agar was cool, it was supplemented with 20 mL of M9 salts (without NH_4Cl, autoclave-sterilized), 0.2 mL of 1M $MgSO_4$ (filter-sterilized), 10 uL of 1M $CaCl_2$ (autoclave-sterilized), 1.5 mL of cortisol solution and 100 uL of ampicillin (100mg/mL). The mixture was poured into petri dishes (20 mL/dish) and the plates subsequently were stored at 4°C.

In a 50mL conical tube the following were added: 24mL of autoclave sterilized ddH_2O, 6 mL of autoclave-sterilized M9 salts (without NH_4Cl), 60 uL of filter-sterilized 1M $MgSO_4$, 450 uL of filter-sterilized cortisol solution (0.033M in ethanol), 3 uL of autoclave-sterilized, 1 M of $CaCl_2$, 0.03 g of NH_4Cl and 30 uL of ampicillin (100 mg/mL).

4.1.b.3 Transformation and growth

The annealed mutagenic pTBSG-COX2 sample and the wild-type pTBSG-COX2 were transformed separately into BL21 competent cells. A mock transformation was also conducted (no plasmid added). Three micro-centrifuge tubes each with 50 uL DH5α cells were thawed on ice, then 2 uL of annealed mutagenic pTBSG-COX2 were added into the one tube and 2 uL wild-type pTBSG-COX2 (251.7 ng/uL) were added to another. The BL21 cells with plasmids were incubated on ice for 30 minutes and then heat shocked at 42°C for 1 minute followed by incubation on ice for 5 minutes. Each tube was then supplemented with 950 uL SOC media and incubated at 37 °C for 2 hours with vigorous shaking (250 rpm). The cells were centrifuged at 3000 rpm for 10 minutes at room temperature and the supernatant was discarded. Then each tube of cells was re-suspended in 1 mL M9-cortisol media and centrifuged at 3000 rpm for 10 minutes at room temperature. After discarding the supernatant, each tube of cells was re-suspended again in 1 mL M9-cortisol media. 500 uL of each tube of cell was plated on the M9-cortisol plate separately and incubated at 37 °C overnight. In addition, 300 uL of the wild type pTBSG-COX2 and annealed mutagenic pTBSG-COX2 transformed cells were added to 10 mL M9-cortisol media separately and incubated at 37 °C overnight with vigorous shaking (250 rpm) - Figure 13

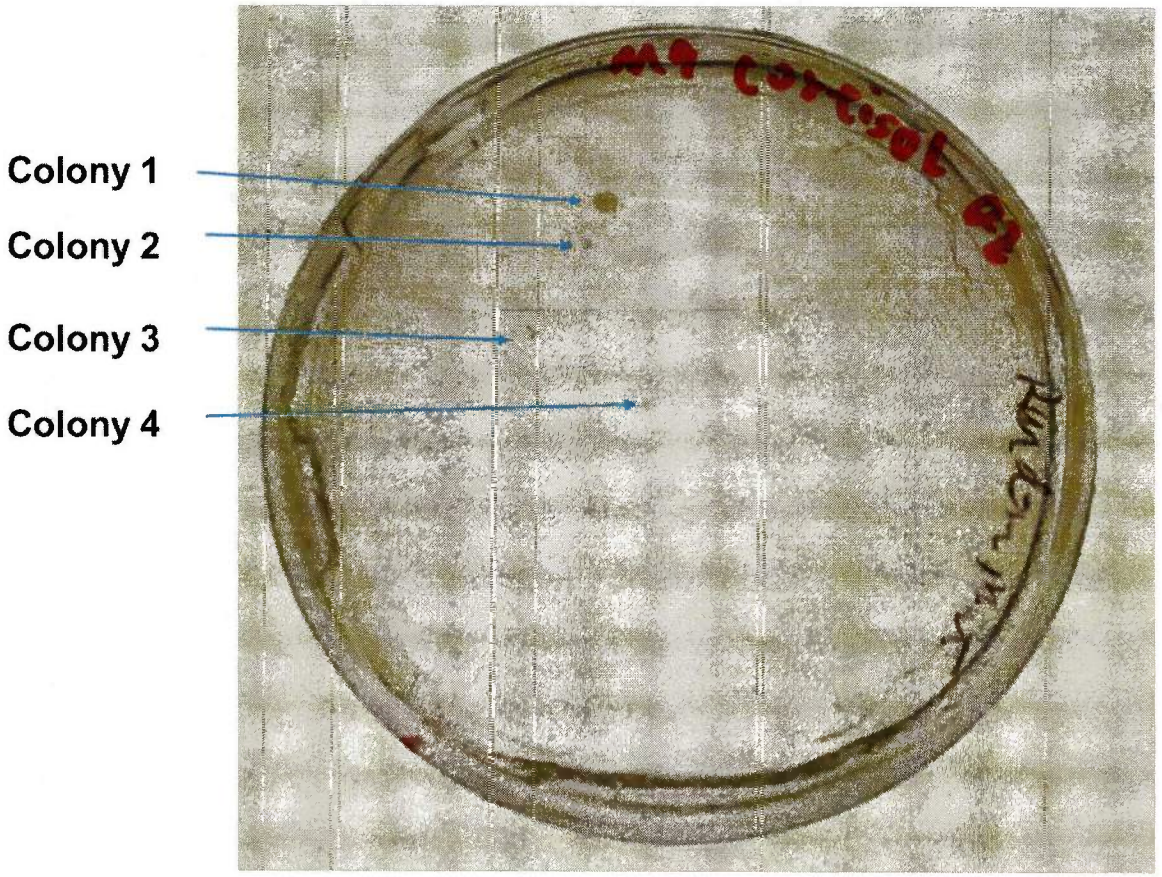


Figure 13 Preliminary results of a PTBSG-3JS8 Mutagenesis Cortisol Screening. Shown is an M9-cortisol plate with randomized 3js8 gene presenting four independent colonies after 2 weeks of growth.

Colonies were picked and seeded into 3 mL overnight culture in LB media containing 100 ug/mL ampicillin. To 1 uL of each overnight culture was mixed with 5uL Lyse and Go PCR Reagent (Thermal Scientific), then 1 uL of each cell lysate was taken for Lyse and Go PCR to screen for insert using the reaction and cycle in Table 3.

Table 3 Mutagenesis Cortisol Screening Protocols

Crimson Taq, 1 reaction	
5x Crimson Buffer	2 uL
dNTP	0.25 uL
10uM F Primer	0.2 uL
10uM R Primer	0.2 uL
DMSO	0.3 uL
Crimson Tap Enzyme	0.2 uL
Cell culture	1 uL
H2O	5.85 uL
Total	10 uL

Temperature	Time	Cycles
95°C	120 s	1
95°C	30 s	
55°C	30 s	30
68°C	75 s	
68°C	300 s	1
4°C	∞	1

Each plasmid was sent for sequencing.

Unfortunately, and despite extensive effort the cortisol directed evolution screens were consistently negative. We implemented an alternative evolutionary strategy of generating mutant libraries using error-prone PCR and DNA shuffling then directly screening for enhanced cortisol oxidation activity in a ninety-six well format. This approach is similar to

that used by Minagawa et al. in isolating mutants of the lactate oxidase. Note that the random nature of the experiment means that multiple experiments performed in exactly the same manner can give dramatically different results. This was also not effective. A random, FACS-based mutant selection method was also tried without success (Figure 14).

In all, we screened thousands of wells, tweaked the processes in numerous ways, and followed a number of false trails. All of this is summarized in the monthly reports. In the end, we found a number of cholesterol oxidase variants that may prove to be very valuable for diagnostic devices and other applications, but we were not able to produce a form of the enzyme that showed significantly more activity and specificity to cortisol then the wild type cholesterol oxidase.

We did identify 18 new mutations that may confer cortisol oxidase activity to the construct. These mutants have been isolated and sequenced, and the identified mutations are summarized in Table 4. No mutation has been observed more than once. These mutants would be promising starting points for future mutational screening to improve catalytic efficiency.

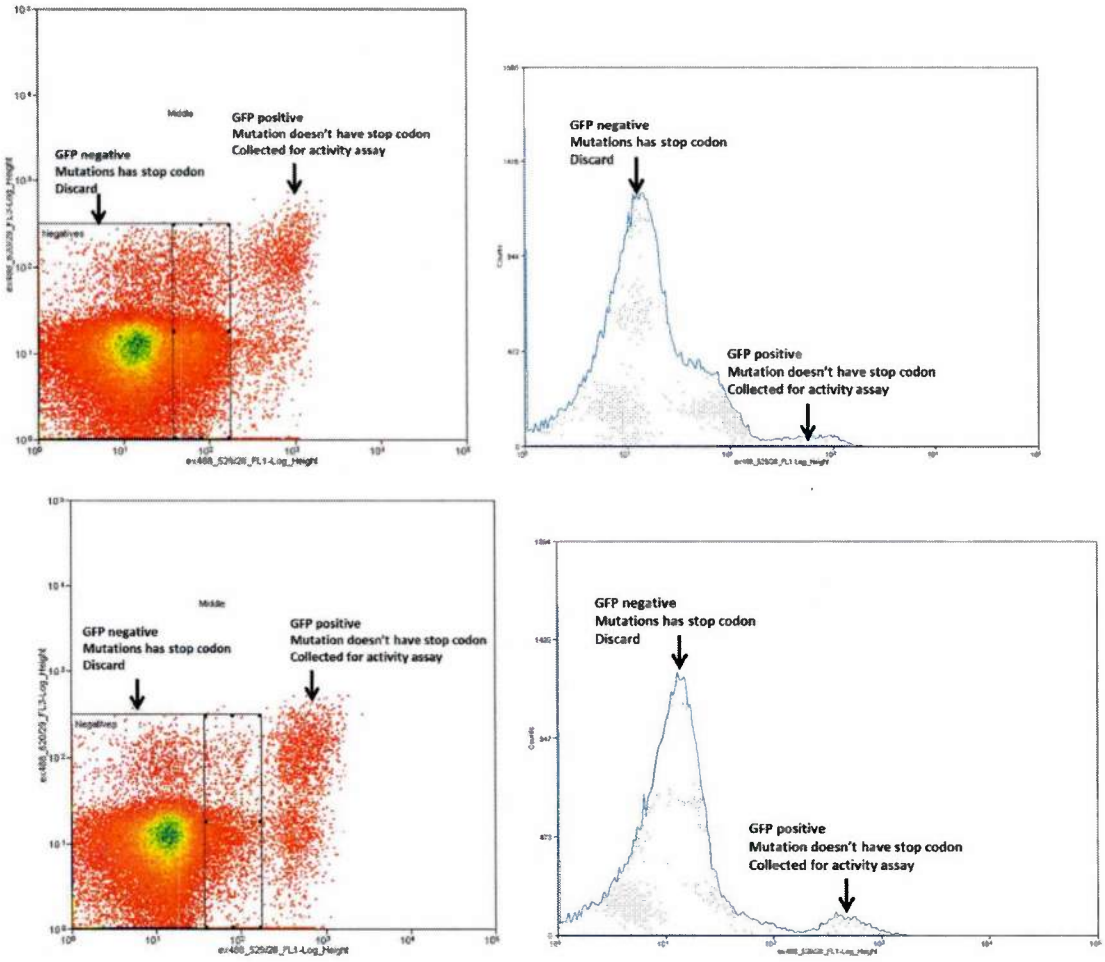


Figure 14 Sample FACS sorting of mutant cholesterol oxidase enzymes to screen for cortisol activity. *Top Left:* Two major populations are distinguished by the presence/absence of fluorescent signal. The population on the left has no fluorescent signal, the mutated 3JS8 insert has stop codon and truncated before the GFP. The population on the right has no fluorescent signal, hence the mutated 3JS8 insert is complete. This is the population that was collected and plated for activity assay. *Top Right:* Histogram of the cell count of *Top Left* sorting based on GFP signal strength. *Bottom Left:* A different sorting experiment yielding similar results to that seen in the *Top Left*. *Bottom Right:* A different sorting experiment yielding similar results to that seen in the *Top Right*. Histogram of the cell count of *Top Left* sorting based on GFP signal strength for *Bottom Left* screen.

Table 4 Summary of cortisol active mutations observed in the COX2 background. Numbering of the mutational site is consistent with the 3JS8 crystal structure available at the RCSB

V45I	T379I	S470P
P197L	F467Y	F226I
L199P	P399L	G252S
K305E	D431Y	V331-
A324P	A488V	-422V
A332S	M62V	G107D

There are two mutations that are of note. The first is M62V and the second is G107D. These two mutations flank the phosphate linker region of the FAD binding pocket (Figure 15), and may exert pressure on the FAD by pulling the co-factor towards these two mutations. In turn, this movement of the FAD makes more room for the cortisol in the active site of the enzyme, thereby allowing the cortisol to find a more favorable orientation for election transfer from the flavin moiety.

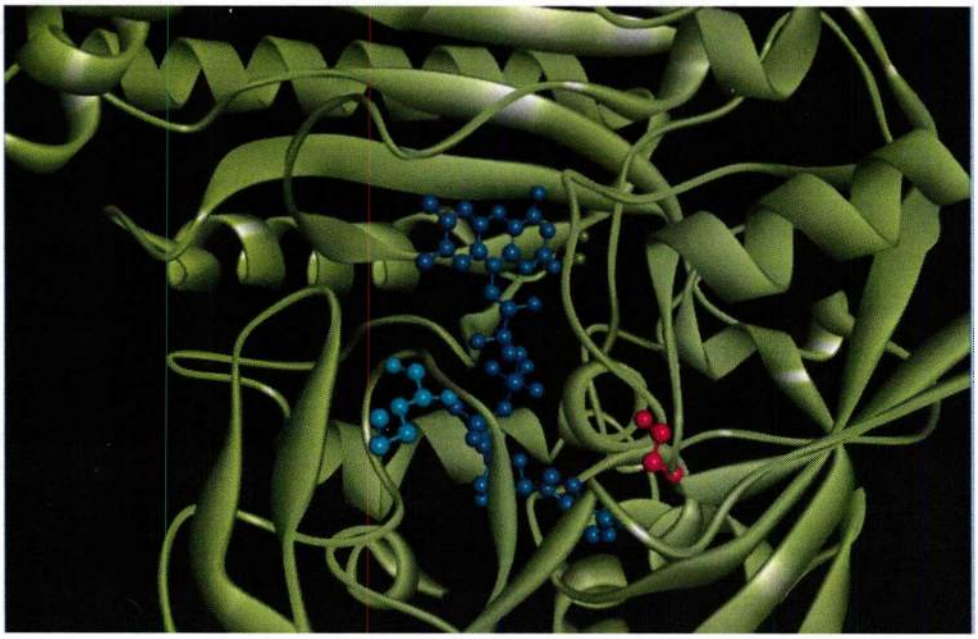


Figure 15 COX2 (3JS8) background crystal structure used for cortisol oxidase directed evolution studies. The FAD prosthetic group is shown in dark blue. Also highlighted are the wild-type residues of the important M62V (light blue) and G107D (red) mutations.

4.2 Large Scale Enzyme Production

There are two potential pathways to optimize the production of active oxidases. The first is to maximize the expression of soluble enzyme. The second is to drive the protein expression to produce inclusion bodies followed by an *in vitro* folding step.

4.2.a Lactate Oxidase Production

A large-scale production protocol for MBP-LOx was determined. The production the MBP-LOx using the enhanced Luria broth protocol followed by refolding of the resultant inclusion bodies is a reliable method for producing this enzyme. The enhanced broth is standard Luria broth (25 g Difco™ LB Broth, Miller, Becton Dickinson #214906 per liter of pure water) with added 19 g Yeast Extract, 2 g Tryptone, 12.54 g of potassium phosphate, dibasic, 2.31 g of potassium phosphate, monobasic. The solution is autoclaved, and then 4 ml glycerol is added prior to induction. Our refolding protocols routinely produce 30-50 mgs of purified protein per liter of cells.

Based on our activity experiments on a biosensor, we identified three sets of conditions that provide well-formulated MBP-LOx enzyme. These conditions are 70% sucrose, 90% sucrose and 90% trehalose. Of these, the 70% sucrose consistently proved to be poor. The 90% sucrose formulation provided the best overall activity, especially as a function of time. This was the formulation that we use for the MBP-LOx final protocol.

We also performed lyophilization studies of MBP-LOX constructs in a variety of glycerol concentrations. Despite the universal deployment of glycerol in protein biochemistry as a cryoprotectant, its use seems to compromise the activity of MBP-LOX when used on a biosensor.

In summary, we have developed protocols to reduce batch-to-batch, assay-to-assay, and technician-to-technician variability. Using these protocols, two 1 liter lots of MBP-Lactate oxidase were manufactured. Both lots exceed targets (protein concentration & activity) set in early 2015. The lactate biosensors produced from both enzyme lots produced responses in the desired sensor range. Multiple assays were performed on the enzyme lots to examine the consistency of the assays. Biosensors produced from these lots show excellent in vitro response, and the assay consistency was acceptable. The results are shown in Figure 16, Figure 17 and Table 5.

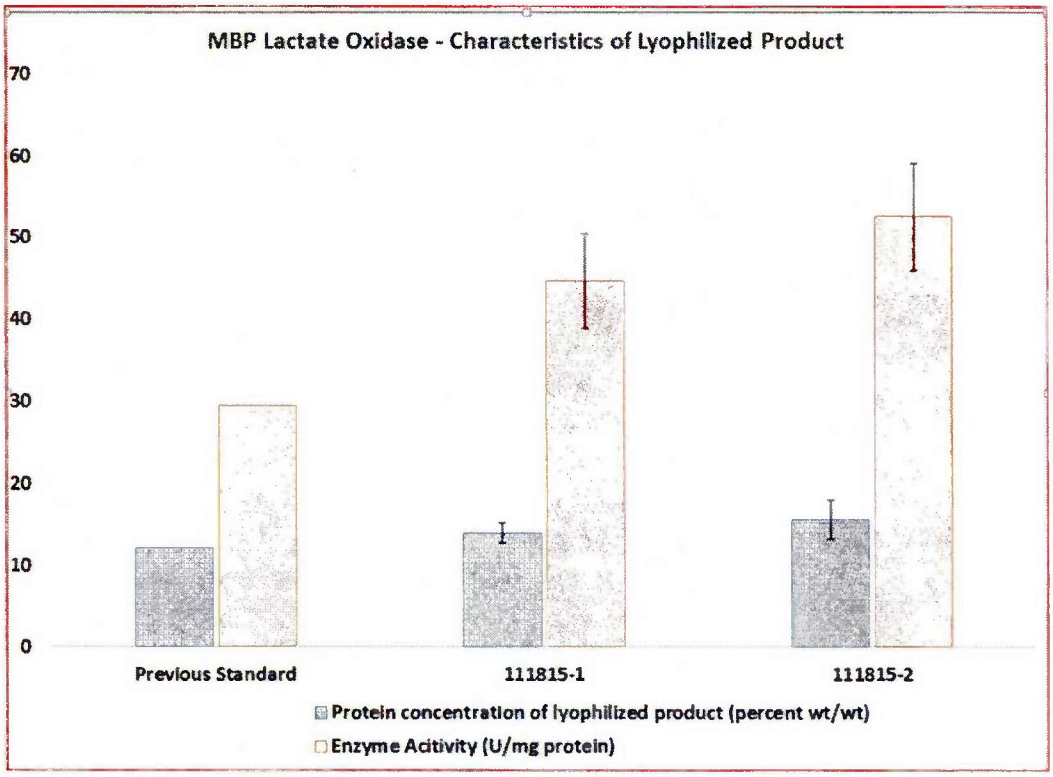


Figure 16 MBP Lactate Oxidase characterization over batches

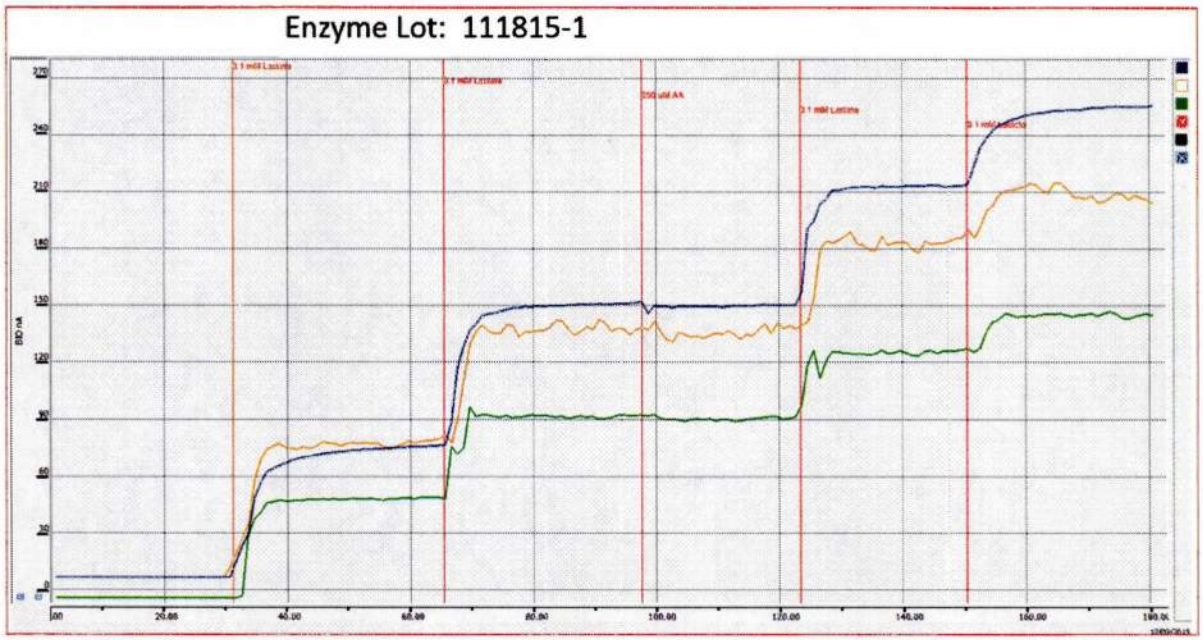


Figure 17 Lactate biosensor calibration curves. Note: These sensors do not have an outer membrane. This is the cause of the non-linearity seen in the response.

Table 5 Results of multiple activity and protein concentration assays.

		ε = 0.0308		ε = 0.058					
111815-1	Protein Concentration	SA (U/mg protein)	SA (U/mg powder)	SA (U/mg protein)	SA (U/mg powder)	Mass (mg)	Vol Water (mL)	[Powder]	[Protein]
Trial 1	12.3%	96.0	11.8	51.0	6.3	1.0	0.114	8.77	1.082
Trial 2	14.8%	90.8	13.4	48.2	7.1	0.9	0.135	6.67	0.984
Trial 3	13.7%	75.9	10.4	40.3	5.5	0.7	0.105	6.67	0.913
Trial 4	15.0%	73.6	11.0	39.1	5.9	0.9	0.135	6.67	1.002
Average	**14.0%**	**84.1**	**11.7**	**44.7**	**6.2**				
sd	1.2%	11.0	1.3	5.8	0.7				
%sd	8.9%	13%	11%	13%	11%				
111815-2	Protein Concentration	SA (U/mg protein)	SA (U/mg powder)	SA (U/mg protein)	SA (U/mg powder)				
Trial 1	15.2%	95.5	14.5	50.7	7.7	0.9	0.212	4.25	0.646
Trial 2	12.6%	92.0	11.6	48.9	6.2	0.9	0.169	5.33	0.673
Trial 3	16.1%	117.3	18.9	62.3	10.0	0.7	0.132	5.30	0.851
Trial 4	18.5%	91.1	16.9	48.4	9.0	0.9	0.169	5.33	0.984
Average	**15.6%**	**99.0**	**15.5**	**52.6**	**8.2**				
sd	2.4%	12.4	3.1	6.6	1.7				
%sd	15.6%	12%	20%	12%	20%				

Over the course of this project, one of the materials used in the MBP-lactate oxidase manufacturing process became unavailable. Diethylaminoethyl cellulose (DEAE-C) was commonly used in ion exchange chromatography for decades but become difficult to source and our vendors said this material would no longer be available in the near future. Therefore, an alternative ion exchanger for protein purification was selected and tested, Diethylaminoethyl sepharose (DEAE-S). MBP-lactate oxidase batch #111815-6 reflects the final results of a complete manufacturing process using DEAE-S. Both the protein concentration and specific activity of the final lyophilized product are very similar to results obtained from previous batches using DEAE-C.

Table 6 MBP Lactate Oxidase batch characterization

Lactate Oxidase Batch 111815-6 Assay report		
	Protein Concentration (% wt/wt)	Specific Activity (U/mg protein)
Assay 1	11.4%	54.8
Assay 2	9.9%	45.4
Assay 3	11.2%	49.5
Average	**10.8%**	**47.5**
SD	*0.8%*	*2.9*

4.2.a.1 Hexamutant Lactate

The hexamutant lactate (LOx-6M) oxidase was also examined, and gave rise to significant soluble expression, especially when using enhanced LB media. We routinely obtain large amounts of soluble MBP-LOx-6M expressed protein (~10-25 mg/L before purification) using enhanced media expression. The his6 tag that is part of the overall construct is slow to bind to Ni(2+) resin, so that the bulk of the protein comes through on the flow-through

under standard conditions. We explored batch binding as part of the protocol and this was effective. For example, we have isolated 3 mg of MBP-LOx-6M with an activity of 48 U/mg starting from 1/4L of post-induction cell material. This activity is the highest ever observed for a lactate oxidase mutant enzyme.

However, desalting the MBP-LOx-6M, a necessary step for the fabrication of biosensors, resulted in a complete loss of activity. This is the first time that we observed this complication. The activity could not be rescued by the re-introduction of FAD (a process that worked for wt-LOx). We looked at expression and resultant activity of soluble MBP-LOx-6M protein from enhanced broth expression at 28, 30, 33, and 37 °C. None of the expressed proteins had substantial activities. We believe that the problem is the lack of cells to produce FAD during expression.

We also examined a wide-range of conditions to express and purify the MBP-LOx-6M protein. While expression provides tens of milligrams of soluble protein, this protein has consistently proven inactive in our hands. Furthermore, the activity of the MBP-LOx-6M protein cannot be rescued with exogeneous FAD added to the media during expression and/or during purification. Based on these results, and given the success we had with MBP-LOx, we halted all exploration on the expression, purification and activation of this protein.

4.2.a.2 Enzyme Stability

Enzyme stability studies of MBP-LOx formulated 90% sucrose and 90% trehalose compared to the commercial enzyme are shown in Figure 18. While the 90% trehalose responds in an equivalent manner to the commercial enzyme over the course of two weeks when fabricated into a biosensor, the 90% sucrose formulation shows superior activity over this time.

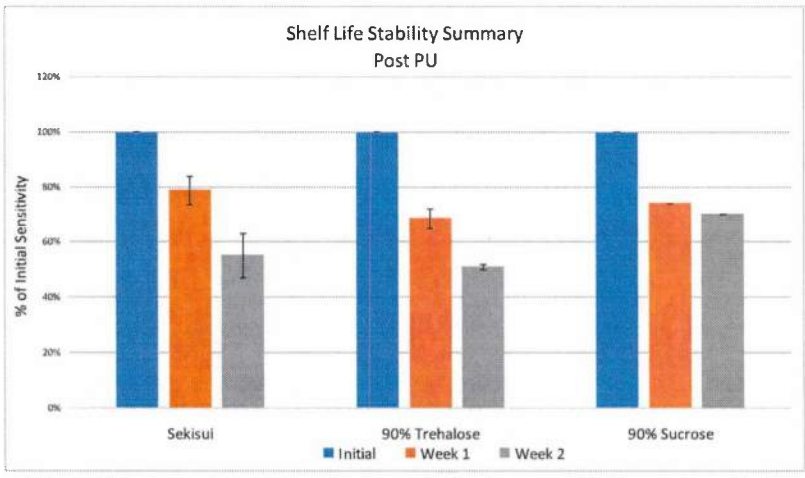


Figure 18 Comparison of activity of commercial Lox (Sikisui), MBP-LOx in 90% trehalose and MBP-LOx in 90% sucrose as a function of time when fabricated into a biosensor (a POC surrogate).

4.2.b Histamine Oxidase Production

The optimal conditions to express histamine oxidase (*Zea mays* diamine oxidase) were investigated. As with the lactate oxidase constructs, the diamine oxidase constructs also failed to yield suitable quantities of soluble protein (Table 7).

Table 7 Soluble Expression of Histamine Oxidase Constructs

	wt-zmDAO 13 °C	wt-zmDAO 22 °C	MBP-zmDAO 13 °C	MBP-zmDAO 22 °C	MBP-zmDAO 37 °C
Yield soluble(mg)	0.2	Dead cells	0.4	5.6	1.9
Specific Activity (U/mg)	ND	NA	ND	< 0.1	< 0.1

In contrast to the lactate oxidase results, the production of histamine oxidase inclusion bodies did not yield significant quantities of material (Table 8). Analysis of the expression products strongly suggest that the proteolysis is occurring prior to inclusion body formation. We believe that the origin of the problem is related to the TEV protease site that is between the fusion protein and the oxidase enzyme. To rectify this problem, we ordered PCR primers to remove the TEV site from these constructs.

Table 8 Yield of Inclusion Bodies for Histamine Oxidase Constructs

Construct	Yield of Inclusion Bodies/ 1 L
wt-zmDAOx	Cells mostly die during expression, < 10mg IB
SUMO-zmDAOx	Cells mostly die during expression, < 10mg IB
MBP-zmDAOx	~44 mg

We performed the mutagenesis on the MBP-DAOX construct to remove the TEV site. As shown in Figure 19, expression of MBP-zmPaOX-(+TEV) @ 15 °C yields essentially no soluble, full-length protein. The largest bands, by intensity, correspond to MBP and wt-zmPaOX. By contrast, expression of MBP-zmPaOX-(-TEV) @ 15 °C shows some full length protein. However, there is still cleavage occurring, as witnessed by the two bands corresponding to MBP and the wt-zmPaOX.

Surprisingly, expression testing at 22 °C showed a completely different profile. Here, expression of MBP-zmPaOX-(-TEV) @ 22 °C shows essentially complete cleavage, as witnessed by the two bands corresponding to MBP and the wt-zmPaOX. Expression of MBP-zmPaOX-(+TEV) @ 22 °C showed some soluble protein in addition to the bands corresponding to MBP and the wt-zmPaOX.

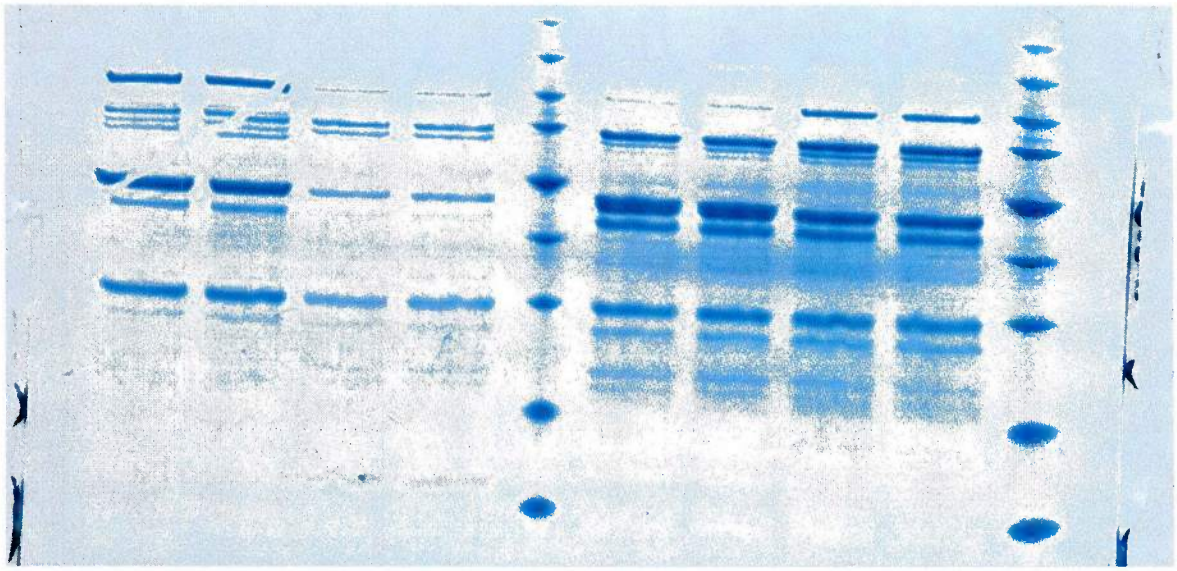


Figure 19 Expression testing as a function of temperature of MBP-zmPaOX-(+TEV) and MBP-zmPaOX-(-TEV). Key from left to right: 1) MBP-zmPaOX (+tev) @ 22c; 2) MBP-zmPaOX (+tev) @ 22c; 3) MBP-zmPaOX (-tev) @ 22c; 4) MBP-zmPaOX (-tev) @ 22c; 5) Molecular Weight Marker; 6) MBP-zmPaOX-(+TEV) @ 15 °C; 7) MBP-zmPaOX-(+TEV) @ 15 °C; 8) MBP-zmPaOX-(-TEV) @ 15 °C; 9) MBP-zmPaOX-(-TEV) @ 15 °C; 10) Molecular Weight Marker

Three constructs (MBP-zmPaOx(+Tev), MBP-zmPaOx(-Tev), and MBP-oxPaOx(+Tev)) were expressed at 37° C and the expressed protein driven into exclusion bodies after induction. The pelleted cells were then stored in a 20 mM Tris-HCl pH 8.0 @ 4° C + 300 mM NaCl buffer at -80° C.

Each preparation was assayed for total protein concentration:

MBP-zmPaOx(+TEV):	31.1 mg/mL	Total protein from 1L = 187 mg
MBP-zmPaOx(-TEV):	18.3 mg/mL	Total protein from 1L = 146 mg
MBP-osPaOx(+TEV):	25.7 mg/mL	Total protein from 1L = 180 mg

Folding of the three constructs was attempted using the protocol for lactate oxidase. This was not successful, and the –TEV construct did not show any significant advantages. We continued to work to identify optimal conditions to allow for the refolding of the inclusion bodies for the MBP-*zm*POA and MBP-*os*POA constructs. This is summarized in Table 9 (pH 6.5), Table 10 (pH 7.4), and Table 11 (pH 8.0).

Table 9 Summary of MBP-zmPOA and MBP-osPOA inclusion body refolding experiments. Proteins (3 ug protein) were assayed using Amplex Red and 200 uM histamine. Because of the pH of the MES buffer, TCEP was used in place of DTT in the folding buffer. Also, inclusion bodies were initially dissolved in 5.5 M Urea Tris HCl pH 8.0 buffer.

						Results	
MBP-zmPOA	ID #	Buffer pH/ Buffer	Protein Conc. (mg/mL)	Urea Conc. Of Folding buffer	Storage Temperature	Protein Conc. (mg/mL)	Activity
	1A25RT	6.5 Mes	20	250 mM	RT	0.16	1.10E-05
	1A50RT	6.5 Mes	20	500 mM	RT	0.17	0
	1A75RT	6.5 Mes	20	750 mM	RT	0.25	0
	1A25	6.5 Mes	20	250 mM	12 °C	0.17	1.50E-06
	1A50	6.5 Mes	20	500 mM	12 °C	0.16	1.00E-05
	1A75	6.5 Mes	20	750 mM	12 °C	0.14	5.90E-06
	1B25RT	6.5 Mes	100	250 mM	RT	0.04	0
	1B50RT	6.5 Mes	100	500 mM	RT	0.04	0
	1B75RT	6.5 Mes	100	750 mM	RT	0.08	0
	1B25	6.5 Mes	100	250 mM	12 °C	0.03	0
	1B50	6.5 Mes	100	500 mM	12 °C	0.06	0
	1B75	6.5 Mes	100	750 mM	12 °C	0.03	0
MBP-osPOA							
	1A25RT	6.5 Mes	20	250 mM	RT	0.14	4.60E-05
	1A50RT	6.5 Mes	20	500 mM	RT	0.15	5.40E-05
	1A75RT	6.5 Mes	20	750 mM	RT	0.16	5.20E-05
	1A25	6.5 Mes	20	250 mM	12 °C	0.11	9.90E-05
	1A50	6.5 Mes	20	500 mM	12 °C	0.17	9.70E-05
	1A75	6.5 Mes	20	750 mM	12 °C	0.19	1.20E-04
	1B25RT	6.5 Mes	100	250 mM	RT	0.17	9.70E-05
	1B50RT	6.5 Mes	100	500 mM	RT	0.16	0
	1B75RT	6.5 Mes	100	750 mM	RT	0.19	0
	1B25	6.5 Mes	100	250 mM	12 °C	0.15	0
	1B50	6.5 Mes	100	500 mM	12 °C	0.16	0
	1B75	6.5 Mes	100	750 mM	12 °C	0.18	0

Table 10 Summary of MBP-zmPOA and MBP-osPOA inclusion body refolding experiments at pH 7.4. Proteins (3 ug protein) were assayed using Amplex Red and 200 uM histamine. DTT was used in the folding buffer. Also, inclusion bodies were initially dissolved in 5.5 M Urea Tris HCl pH 8.0 buffer.

zmPOA and osPOA Folding Screen						
zmPOA	pH of Buffer	Protein Conc.	Urea Conc. Of Folding buffer	Stored at Room temp or 12°C	Protein Conc.	Activity
2A25RT	7.4 Tris	20ug/mL	250mM	Room temp	.17 mg/mL	3.50E-05
2A50RT	7.4 Tris	20ug/mL	500mM	Room temp	.12 mg/mL	4.90E-05
2A75RT	7.4 Tris	20ug/mL	750mM	Room temp	.12 mg/mL	6.70E-05
2A25	7.4 Tris	20ug/mL	250mM	12°C	.15 mg/mL	2.10E-05
2A50	7.4 Tris	20ug/mL	500mM	12°C	.17 mg/mL	2.90E-05
2A75	7.4 Tris	20ug/mL	750mM	12°C	.17 mg/mL	4.40E-05
2B25RT	7.4 Tris	100ug/mL	250mM	Room temp	.35 mg/mL	4.70E-05
2B50RT	7.4 Tris	100ug/mL	500mM	Room temp	no result due to technician error	
2B75RT	7.4 Tris	100ug/mL	750mM	Room temp	no result due to technician error	
2B25	7.4 Tris	100ug/mL	250mM	12°C	.51 mg/mL	1.80E-06
2B50	7.4 Tris	100ug/mL	500mM	12°C	.5 mg/mL	2.90E-06
2B75	7.4 Tris	100ug/mL	750mM	12°C	.46 mg/mL	2.00E-06
osPOA						
2A25RT	7.4 Tris	20ug/mL	250mM	Room temp	no result due to technician error	
2A50RT	7.4 Tris	20ug/mL	500mM	Room temp	.16 mg/mL	1.40E-05
2A75RT	7.4 Tris	20ug/mL	750mM	Room temp	.16 mg/mL	1.70E-05
2A25	7.4 Tris	20ug/mL	250mM	12°C	.22 mg/mL	7.60E-06
2A50	7.4 Tris	20ug/mL	500mM	12°C	.22 mg/mL	4.40E-06
2A75	7.4 Tris	20ug/mL	750mM	12°C	.24 mg/mL	1.30E-05
2B25RT	7.4 Tris	100ug/mL	250mM	Room temp	.28 mg/mL	7.10E-05
2B50RT	7.4 Tris	100ug/mL	500mM	Room temp	.35 mg/mL	5.90E-05
2B75RT	7.4 Tris	100ug/mL	750mM	Room temp	.44 mg/mL	5.50E-05
2B25	7.4 Tris	100ug/mL	250mM	12°C	.42 mg/mL	0
2B50	7.4 Tris	100ug/mL	500mM	12°C	0.35 mg/mL	7.30E-06
2B75	7.4 Tris	100ug/mL	750mM	12°C	.47 mg/mL	7.60E-07

The results are about the same at pH 8.0 and pH 7.4 for the MBP-*zm*POA, and both are superior to those shown at pH 6.5. The refolding of the MBP-*os*POA constructs are significantly poorer at pH 8.0 compared to pH 7.4

Table 11 Summary of MBP-zmPOA and MBP-osPOA inclusion body refolding experiments at pH 8.0. Proteins (3 ug protein) were assayed using Amplex Red and 200 uM histamine. DTT was used in the folding buffer. Also, inclusion bodies were initially dissolved in 5.5 M Urea Tris HCl pH 8.0 buffer.

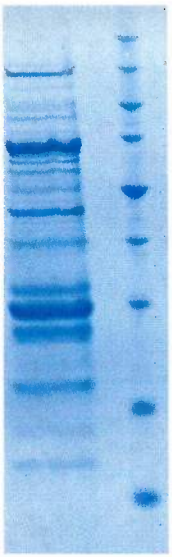
					Results	
zmPOA	pH of Buffer	Protein Conc.	Urea Conc. Of Folding buffer	Stored at Room temp or 12°C	Protein Conc.	Activity
3A25RT	8.0 Tris	20ug/mL	250mM	Room temp	0.03 mg/mL	2.95E-05
3A50RT	8.0 Tris	20ug/mL	500mM	Room temp	0.19 mg/mL	5.74E-05
3A75RT	8.0 Tris	20ug/mL	750mM	Room temp	0.2 mg/mL	2.56E-05
3A25	8.0 Tris	20ug/mL	250mM	12°C	0.15 mg/mL	1.87E-05
3A50	8.0 Tris	20ug/mL	500mM	12°C	0.19 mg/mL	3.81E-05
3A75	8.0 Tris	20ug/mL	750mM	12°C	0.17 mg/mL	3.13E-05
3B25RT	8.0 Tris	100ug/mL	250mM	Room temp	0.15 mg/mL	2.82E-05
3B50RT	8.0 Tris	100ug/mL	500mM	Room temp	0.19 mg/mL	5.27E-05
3B75RT	8.0 Tris	100ug/mL	750mM	Room temp	0.17 mg/mL	2.07E-05
3B25	8.0 Tris	100ug/mL	250mM	12°C	0.12 mg/mL	1.20E-05
3B50	8.0 Tris	100ug/mL	500mM	12°C	0.13 mg/mL	2.40E-05
3B75	8.0 Tris	100ug/mL	750mM	12°C	0.11 mg/mL	2.00E-05
osPOA						
3A25RT	8.0 Tris	20ug/mL	250mM	Room temp	0.15 mg/mL	3.84E-06
3A50RT	8.0 Tris	20ug/mL	500mM	Room temp	0.15 mg/mL	9.67E-05
3A75RT	8.0 Tris	20ug/mL	750mM	Room temp	0.14 mg/mL	9.66E-06
3A25	8.0 Tris	20ug/mL	250mM	12°C	0.17 mg/mL	9.58E-07
3A50	8.0 Tris	20ug/mL	500mM	12°C	0.18 mg/mL	1.29E-06
3A75	8.0 Tris	20ug/mL	750mM	12°C	0.21 mg/mL	3.66E-06
3B25RT	8.0 Tris	100ug/mL	250mM	Room temp	0.17 mg/mL	1.10E-05
3B50RT	8.0 Tris	100ug/mL	500mM	Room temp	0.18 mg/mL	1.48E-05
3B75RT	8.0 Tris	100ug/mL	750mM	Room temp	0.21 mg/mL	7.08E-06
3B25	8.0 Tris	100ug/mL	250mM	12°C	0.13 mg/mL	7.90E-08
3B50	8.0 Tris	100ug/mL	500mM	12°C	0.15 mg/mL	4.00E-06
3B75	8.0 Tris	100ug/mL	750mM	12°C	0.15 mg/mL	2.30E-07

Based on these less than satisfactory results, two new conditions were explored in addition to the trials already conducted, 1) varying the concentration of folding buffer components that had not yet been screened (glycerol, arginine, dithiothreitol), and 2) adding the molecular chaperone GroEL (Hsp60) plus ATP to the folding buffer. These conditions were examined via dilution folding vs dialysis folding. Up to equimolar concentrations of the Hsp60 with the oxidase was included during the folding process along with varying glycerol concentrations. Note that Hsp60 is stable in 4 M urea so it could be added early in the folding process. Up to equimolar concentrations of the Hsp60 with the inclusion bodies were attempted without success.

Independently of the expanded refolding screen, we also examined the expression of MBP-zmPOA and MBP-osPOA constructs in *pseudomonas* and *lactococcus* expression systems. It is known that these strains have completely different refolding machinery inside the cells compared to *E. coli*. It has been shown in a number of fusion systems for which there is no natural folding pathway, that expression in *pseudomonas* or *lactococcus* can result in soluble protein. This is especially true for those constructs that only yield inclusion bodies in *E. coli*. This enhancement of refolding is often attributed to different lipids that are naturally occurring in each of these strains.

A shuttle vector for *pseudomonas* (the gene becomes chromosomally inserted) and an expression plasmid for *lactococcus* were obtained as well as suitable host cells allowing IPTG induction for both systems. Subcloning into the vectors starting with the MBP-

zmPOA gene was carried out. Following induction, cultures were screened for soluble protein expression and associated enzymatic activity.



Initially, we had difficulty optimizing the electroporation process. This was corrected by replacing the electroporator probe. Expression of the MBP-zmPOA construct in a pseudomonas expression system (*Pseudomonas Areginosa* or *P. areg*) was quantified. Transformants from proper electroporation grew well with 200 ug/uL carbenecillin. A 1L expression was performed in enriched LB media (50 g low salt LB mix + 89mM pi 7.5 + 4 mg/mL glycerol) for 3 hours @ 32C using 2mM IPTG. The lysate from these cells was bright yellow suggesting the presence of FAD-bound protein. Ni-NTA purification of the lysate from these cells yielded ~8 mg of colorless protein @ 1.7 mg/mL. (Figure 20).

Figure 20 SDS-PAGE gel of MBP-zmPOA produced in *P. areg*. The sample showed no activity, and does not show a significant band at about 100 kDa. Marker mass from top down: 225, 150, 100, 75, 50, 35, 25, 10 kDa.

The prominent upper band runs below the 100kDa marker, which is inconsistent with the predicted molecular weight of the MBP-zmPaOX construct (56.3+ 45.0 = 103.3 kDa). No significant enzymatic activity could be detected in the eluate in 1 hr using 100 ug protein. This corresponds to < 1 E -6 U / mg of specific activity. No target protein bands were present in either soluble or insoluble fractions by SDS-PAGE. PCR screening of wt-zmPOA transformants indicated the presence of the constructs, whereas PCR screening of the MBP-zmPOA transformants failed to detect the construct. Thus, the MBP-zmPOA transformants seemed to have lost the plasmid.

After this series of set-backs, we finally achieved excellent results using enhanced Luria broth (described in 4.2a). We looked at both MBP-zmPOA and MBP-osPOA in standard and enhanced LB media. We were able to produce almost 22 mg of MBP-zmPOA from 1 liter of soluble expression. This is the first example of soluble expression of this material at reasonable levels. Historically, expression levels were about 0.25 mg/L. Thus, this constitutes an almost 100-fold improvement in expression efficiency. While there are still details to be worked out, the fact that we are observing soluble expression of a histamine oxidase enzyme at reasonable yields is a major accomplishment. This material also shows very slow binding to the Ni(2+) resin and initial purifications yield very little purified protein as most of the protein was in the flow-through. Furthermore, the interaction with the Ni(2+) resin is atypical. Usually, we see the resin remain clear or take on a yellow color, which is indicative of binding (the yellow is characteristic of an FAD-dependent protein with a high FAD cofactor residency).

4.3 Lactate Oxidase Validation

The MBP-LOX, when used in the fabrication of biosensors gave rise to good responses in most of the biosensors tested (Table 12). Testing yielded biosensors fabricated from the various MBP-LOx formulations that are comparable to those seen for biosensors fabricated from commercial enzyme. It is noted that the biosensors fabricated from formulations containing sucrose show lower overall sensitivity compared to the trehalose formulations. This may be due, in part, to the error in correcting for protein concentration during the formulation process.

Table 12 Summary of pre- and post-polyurethane responses for biosensors fabricated from wild-type lactate oxidase (Sekisui, a commercial source), MPB-LOx formulated with trehalose, and MPB-LOx formulated with sucrose.

Sensor	Enzyme Type and Formulation	Pre-polyurethane Lactate Response (nA)	Post-polyurethane Lactate Response (nA)
DM08	Commercial (non-MBP)	95.8	3.67
DM09	Commercial (non-MBP)	94.3	6.81
DM10	Commercial (non-MBP)	88.8	4.04
DM11	MBP-LOx, 2% Trehalose	109.7	7.27
DM12	MBP-LOx, 2% Trehalose	81.2	11.14
DM13	MBP-LOx, 2% Trehalose	74.9	0.03
DM14	MBP-LOx, 2% Sucrose	70.7	0.91
DM15	MBP-LOx, 2% Sucrose	70.6	2.14
DM18	MBP-LOx, 2% Sucrose	60.5	6.51

Lactate biosensors can be routinely manufactured using the MBP-lactate oxidase enzyme (90% sucrose formulation). Sensitivity testing of the MBP-lactate oxidase biosensors found that they meet or exceed the performance biosensors made with commercial (Sekisui) lactate oxidase in terms of limit of detection, sensitivity and linearity.

Our proprietary fabrication process of a biosensor can, in some instances, rescue the activity of an oxidase enzyme that has lost co-factor. Thus, we built some lactate biosensors from the MBP-LOx-6M which showed no activity. As shown in Figure 21, the fabrication process did not rescue the enzyme and consequently the biosensor response (shown in red) when compared to the control shown in blue.

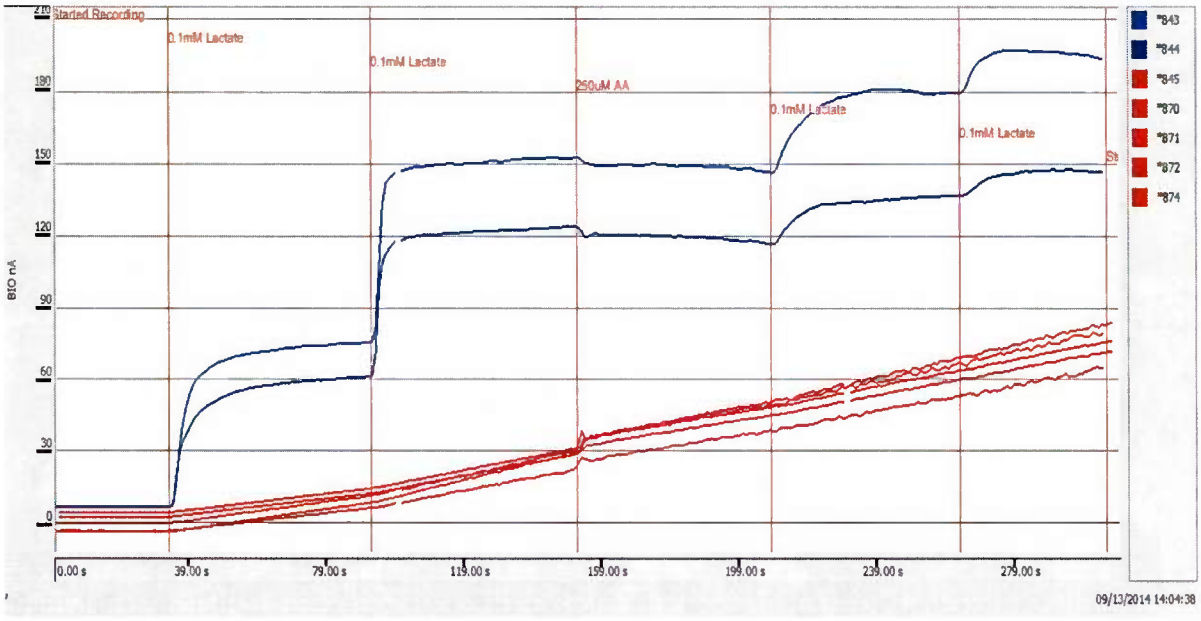


Figure 21 The rescue of inactive MBP-LOx-6M enzyme does not occur during the biosensor fabricated process. Traces of biosensors made from MBP-LOx-6M (Red) remain inactive in comparison to controls (blue).

4.3.a Shelf-Life Stability

Shelf-life stability testing of the lactate biosensor was done on biosensors made using the MBP-lactate oxidase. As outlined in the Methods Section, biosensors were tested under 5 storage conditions: 0 °C, 4 °C in buffer, 4 °C in air, Room temperature (RT) in buffer and RT in air. Sensor performance was tested over a three week period on a weekly basis. In this testing paradigm, no sensor was tested more than twice (initial test plus a re-test at the appropriate time point). This is because repeated re-testing can affect the sensitivity and selectivity of the biosensor and we wanted to make sure that this variable was removed. Figure 22 shows an example of the level of variability within a single batch of sensors under one testing condition. Figure 23 is a summary of shelf-life for all storage conditions tested. Over 80 CNS diagnostic devices were tested revealing good to great stability of the devices in all storage conditions.

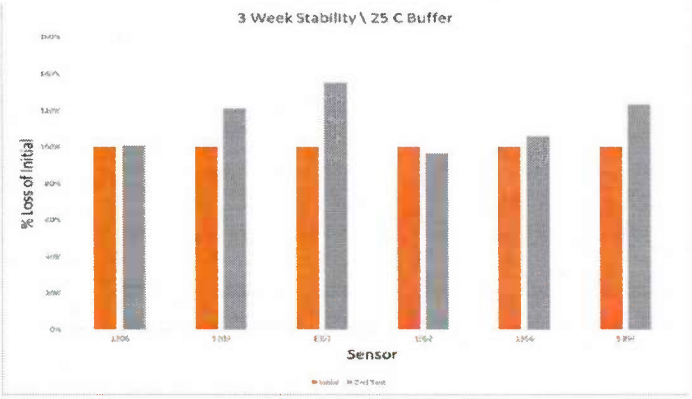


Figure 22 Stability plot of biosensors fabricated with MBP-LOx after 3 weeks in buffered solution.

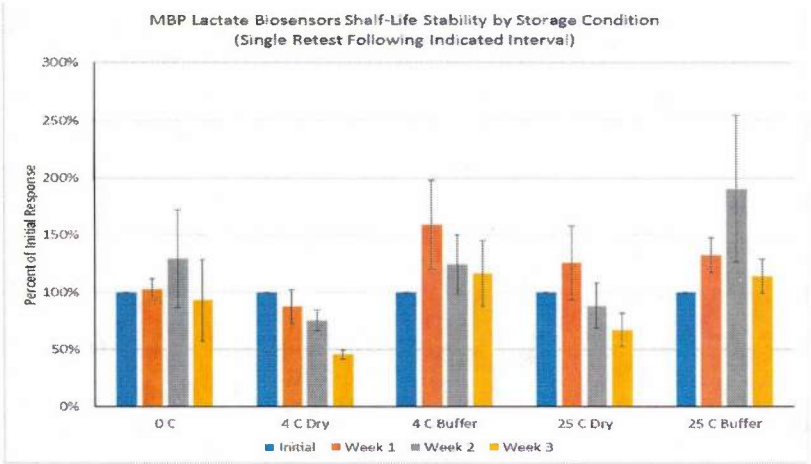


Figure 23 Summary of shelf life performance as a function of storage condition for CNS diagnostic devices that use MBP-lactate oxidase enzyme.

In addition to the shelf-life testing paradigm described above, we expanded the scope of this testing to include weekly retesting of all sensor groups after week 3. These data are summarized in Figure 24. For clarity, the blue line in each group is the initial Day 0 test,

the orange line is the first retest at Week 3. The remaining bars are weekly retests after that period. These data further demonstrate the shelf stability of the MBP-lactate oxidase enzyme.

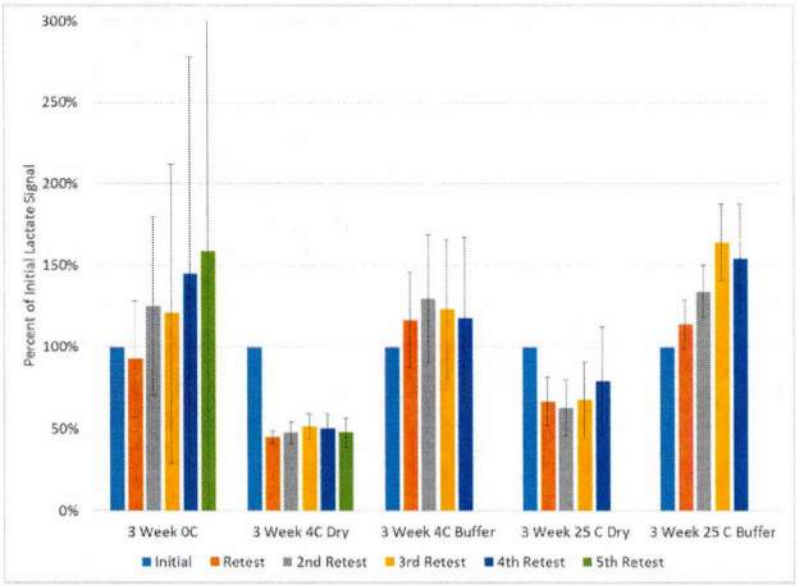


Figure 24 Stability plot of the biosensors fabricated with MBP-lactate oxidase after multiple retests.

Our within group variability is exceedingly good as can be seen in Figure 25. Note that the retesting takes place over an 11 week period. Biosensors made with the commercial oxidase have a shelf life considerable less than 11 weeks.

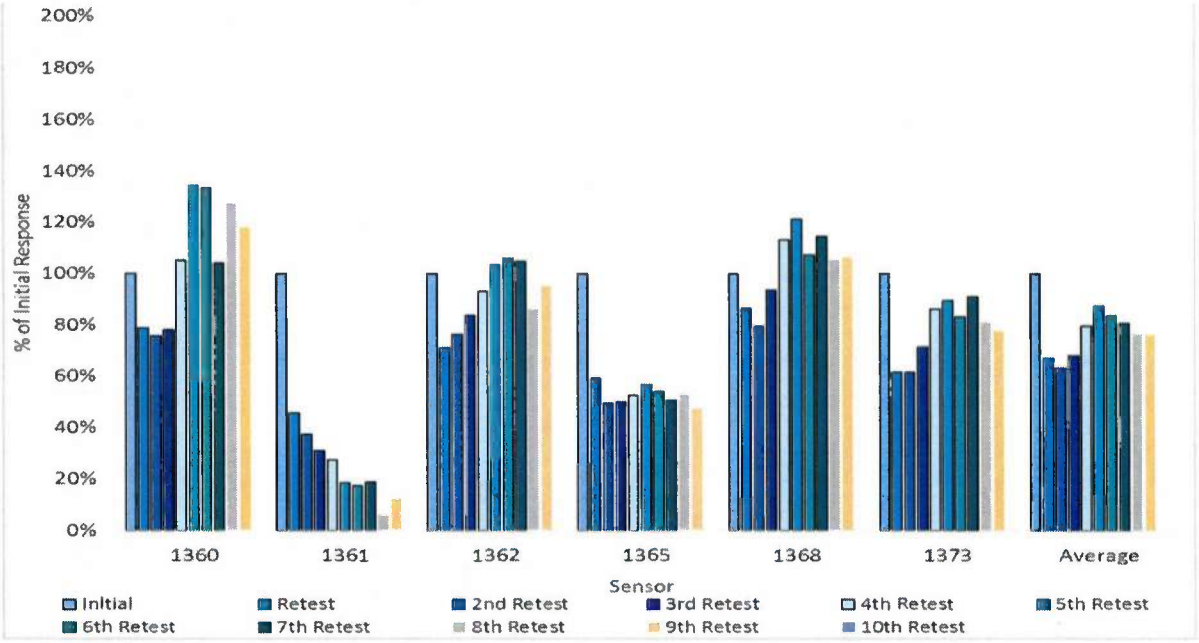


Figure 25 Summary of shelf life performance at room temperature for biosensors made with MBP-lactate oxidase enzyme. Note that despite repeated weekly retesting, the average response of the devices is maintained for an extended period of time. This trend was observed for all the storage conditions investigated.

4.3.b Subcutaneous Biosensor Fabrication

Lactate biosensors can be routinely fabricated from both the commercial enzyme source (Sekisui Diagnostics) and the Pinnacle developed MBP-LOx (90% sucrose formulation) for the purposes of subcutaneous implantation. These sensors show excellent *in vitro* response. In one experiment, three of each sensor type were manufactured and tested prior to application of the outer, polyurethane membrane (Figure 26). Figure 27 shows the final test (post polyurethane application and attachment to subcutaneous implantation substrate). This particular example shows lower post-PU sensitivity for the MBP-Lox sensor, but this is due to variations in the thickness and structure of the applied polyurethane, not to the activity of the immobilized enzyme.

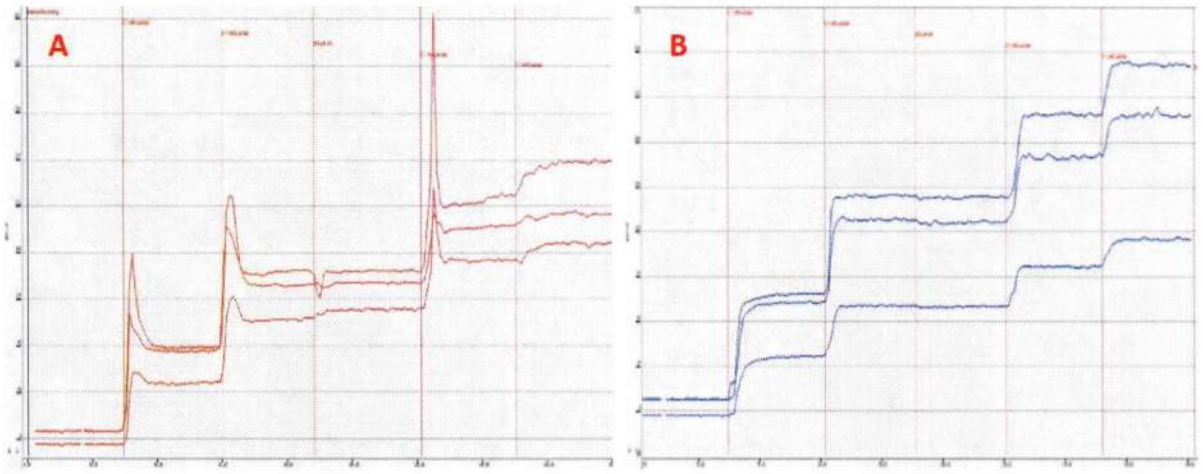


Figure 26 Standard pre-polyurethane *in vitro* calibration of lactate biosensors manufactured with (A) commercial Sekisui lactate oxidase (red traces) and (B) Pinnacle MBP-lactate oxidase (blue traces).

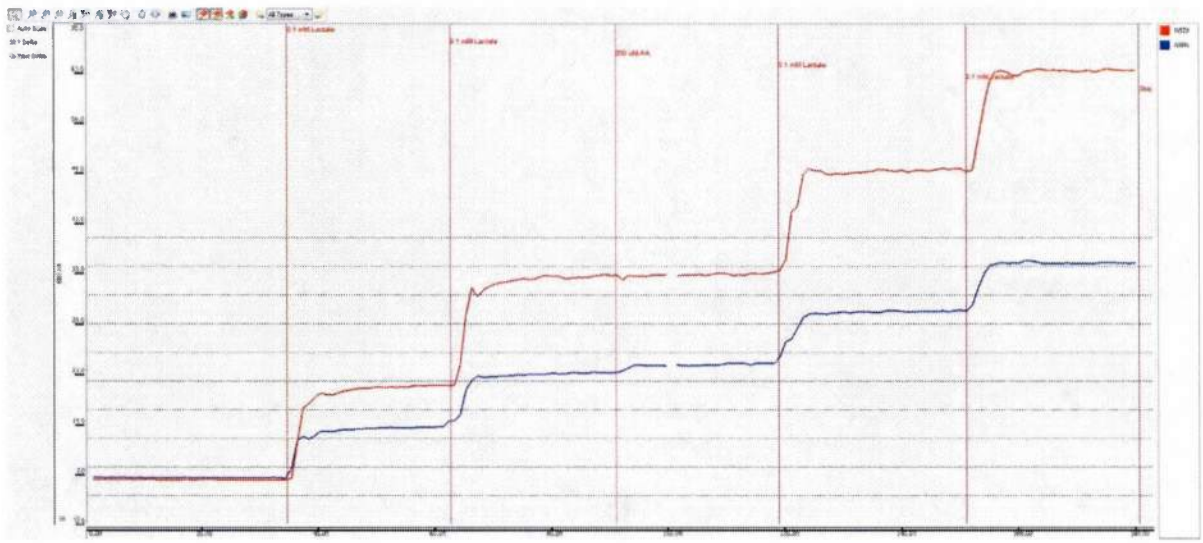


Figure 27 *In vitro* calibration curve for the two lactate biosensors manufactured for subcutaneous implantation. The lactate biosensor manufactured using commercial (Sekisui) lactate oxidase is the red trace. The blue trace is the lactate biosensors manufactured using the in-house developed MBP-lactate oxidase (90% sucrose formulation).

4.3.c. CNS *In Vivo* Testing

To perform the *in vivo* studies a specially made dual guide cannula (Figure 28) was fashioned such that a biosensor and an injection needle could be lowered into brain within very close proximity to each other. A Pinnacle 8164 Wireless Potentiostat was used interface with the biosensor. This particular device was necessary because it is battery operated and the motorized injector (Quintessential Stereotaxic Injector, Stoelting) created a significant amount of electrical noise on the AC powered Pinnacle 8102 Desktop Potentiostat. By using the wireless, battery powered potentiostat, we were able to monitor

the biosensor signal while the injector was powered on and in use without any issues. Figure 29 shows the response of the implanted biosensor to a local injection of l-lactate in the brain. This particular biosensor was manufactured using MBP-Lactate oxidase formulated with 85% trehalose.

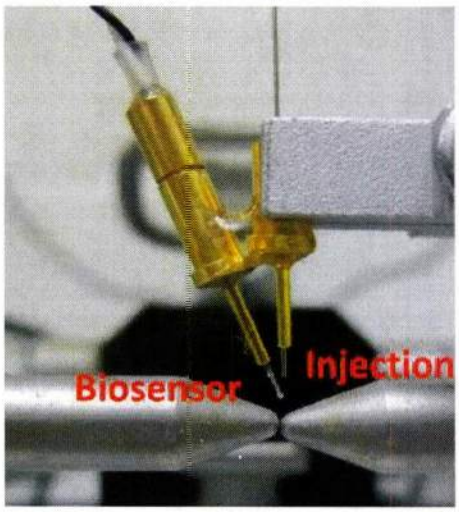


Figure 28 A dual cannula fashioned to accommodate the biosensor and injection needle in close proximity.

There was increased noise on the biosensor trace that was a direct result of the motorized injector being powered on. Also, during injections we encountered issues with the motorized injector. In one case the programmed volume for delivery was not correctly delivered. The vendor was contacted to address these issues of incorrect volume delivery in future experiments as well as the problem of the motorized system being a source of electrical interference for the desktop.

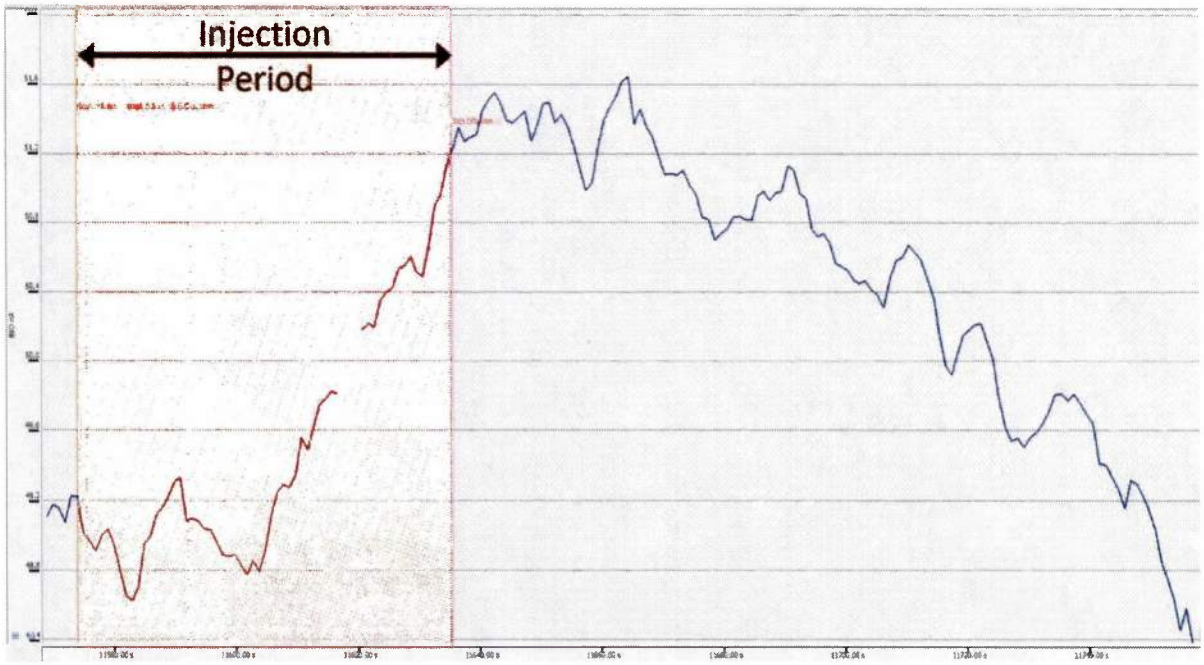


Figure 29 Biosensor response to a 0.5uL bolus of 0.5 M L-lactate. The injection period (1 minute) is shown overlaid in red. The peak height of the biosensor corresponds to an increase in lactate concentration of approximately 0.06 mM.

Our *in vivo* characterization leverages the physiological relationship between sleep/wake states and changes in brain (pre-frontal cortex) lactate concentration to gauge the performance of the diagnostic device over an extended period of time. It has been demonstrated that when an animal transitions from a sleeping to a wakeful state brain lactate levels rise and then fall when an animal transitions from a waking state to a sleep state. To use this relationship as an evaluation tool, we implanted rats with lactate biosensors paired with Pinnacle's wireless telemetry devices to monitor brain lactate changes using biosensors manufactured with either MBP-lactate oxidase or commercially obtained (Sekisui) lactate oxidase. These studies have shown the sensors using MBP-Lactate oxidase to have better overall performance, achieving a stable, in vivo baseline faster and lasting significantly longer than the sensors made using commercial enzyme (Figure 30).

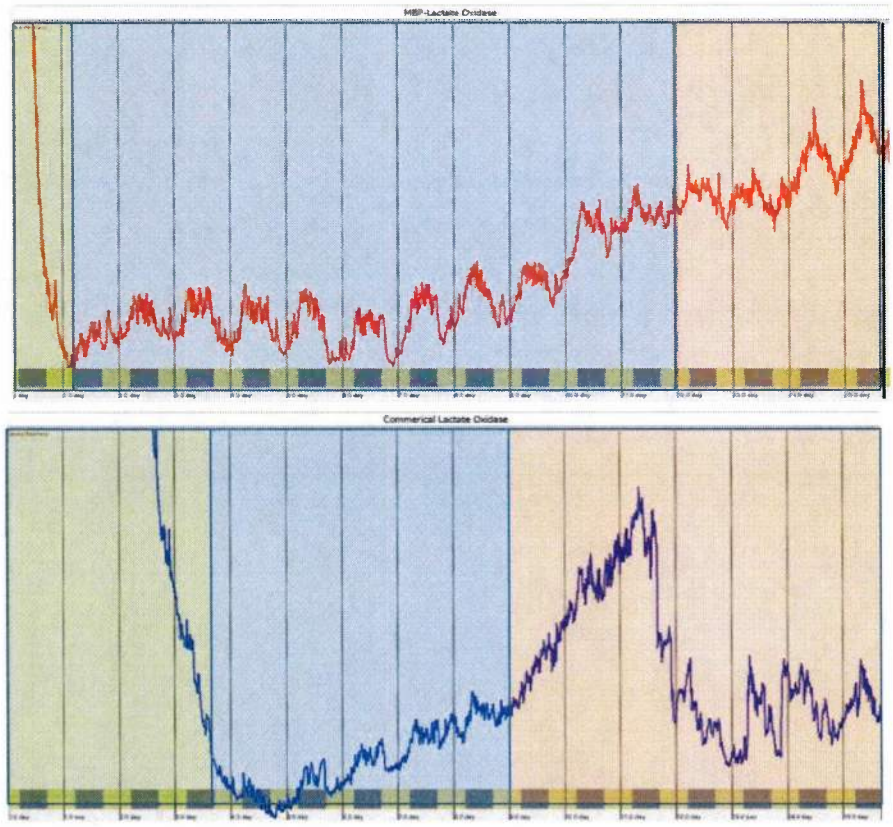


Figure 30 Fifteen day *In vivo* traces of CNS diagnostic devices using MBP-lactate oxidase (upper trace) and commercial lactate oxidase (lower trace). Both traces have been overlaid with shaded regions to denote the "run-in period" (green), the expected trace for performance monitoring (blue) and the period of lifetime where the device feedback has become unreliable with regard to lactate monitoring of sleep/wake transitions (red).

In the previous set of experiments described, the commercial lactate oxidase enzyme and the MBP-lactate oxidase developed in this effort were tested in separate animals. In another experiment, rats were implanted with two sensors in the pre-frontal cortex (contralateral placement). One of the sensors was manufactured using the MBP-Lactate oxidase and the other was manufactured using commercially obtained lactate oxidase. Again, the sensors using MBP-Lactate oxidase had better overall performance, achieving a stable, *in vivo* baseline faster and lasting significantly longer than the sensors prepared using commercial enzyme (Figure 31). Further comparisons showed that the MBP-lactate oxidase biosensors maintain a more stable amplitude of response over a long period of time (eight days) while the signal amplitude of the commercial enzyme based biosensor decreased noticeably over the same time period.

The MBP-lactate oxidase devices continue to exhibit a better level of baseline stability over time than the commercial enzyme devices (Figure 32). Furthermore, MBP-lactate oxidase devices also exhibit a higher signal amplitude over time. Unfortunately this round of in vivo experiments ended prematurely when the implanted head-stages became separated from the animals at day 16 and 18 respectively.

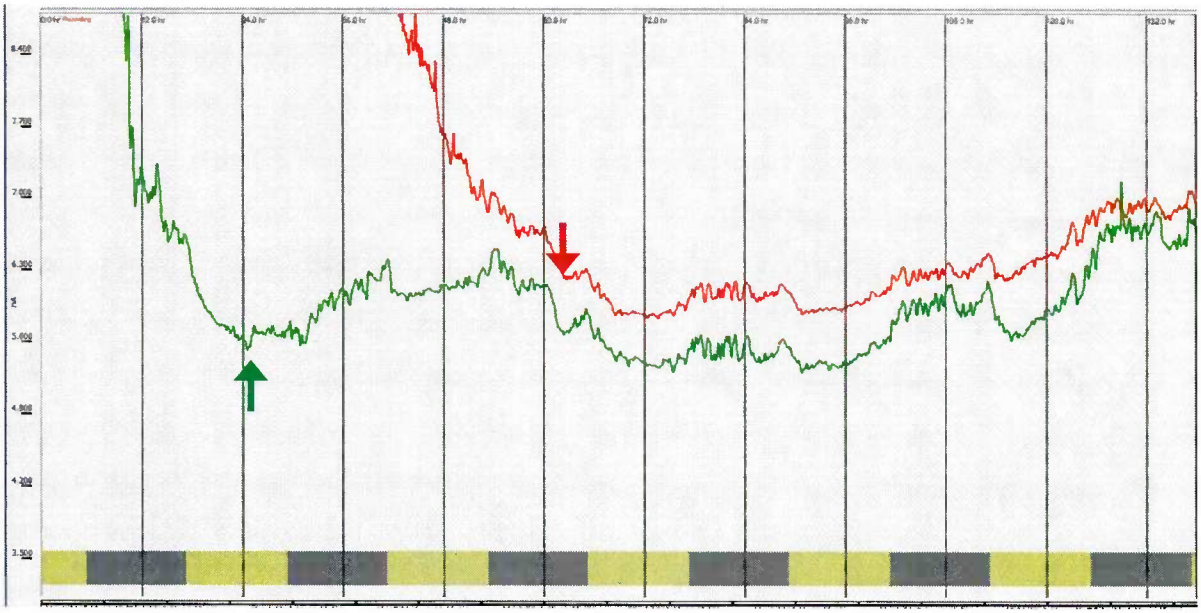


Figure 31 Contralateral implantation of Lactate Oxidase biosensors manufactured with MBP-lactate oxidase (green trace) and commercially obtained lactate oxidase (Sekisui – red trace). The MBP-lactate oxidase biosensors achieved a stable baseline current approximately within the first 24 hours from implantation (green arrow), while the commercial enzyme based biosensor did not reach a steady baseline until after 48-72 hours after implantation. (Filters applied: 60pt median filter, 10pt mean filter, 10pt binning).

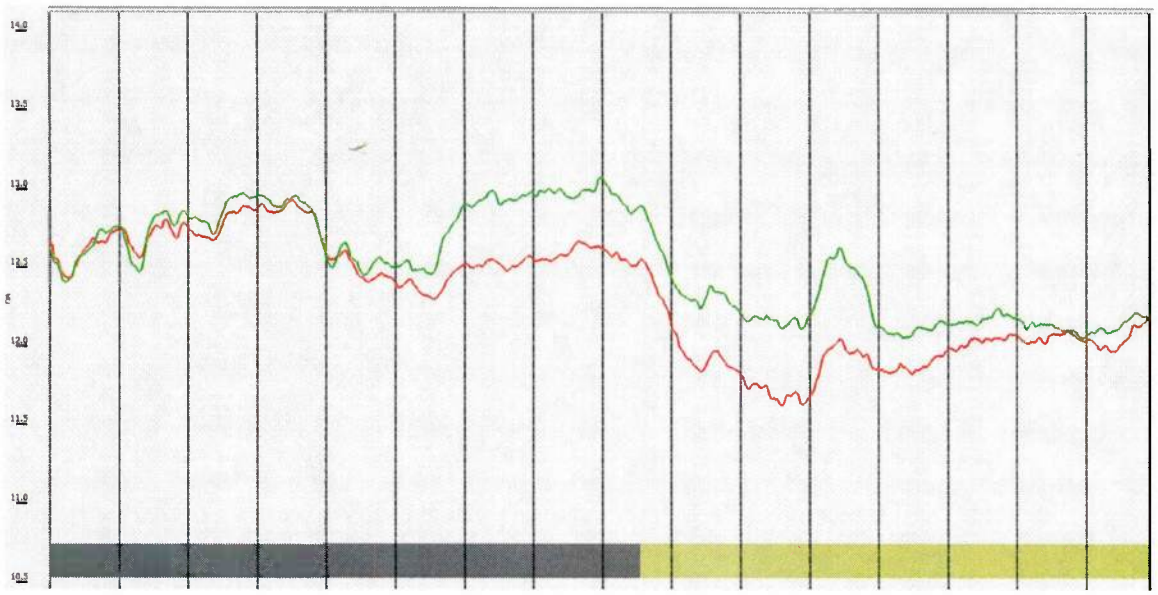


Figure 32 Contralateral implantation of Lactate Oxidase biosensors manufactured with MBP-lactate oxidase (green trace) and commercially obtained lactate oxidase (Sekisui – red trace). The MBP-lactate oxidase biosensors had more stability associated with its baseline. (Filters applied: 60pt median filter, 10pt mean filter, 10pt binning)

4.3.d Subcutaneous In Vivo Testing

Lactate biosensor were also fashioned from MBP-LOx for the purposes of subcutaneous implantation. These sensors showed good *in vitro* response, Figure 33. They were implanted subcutaneously in male, Sprague Dawley rats in order to demonstrate the use of the enzyme as another point of care device (as opposed to its use in the brain). In this deployment, the lactate biosensors were manufactured on a modified sensor substrate that allowed for the sensing device to be secured within the subcutaneous layer as opposed to the typical intracerebral deployment (Figure 34).

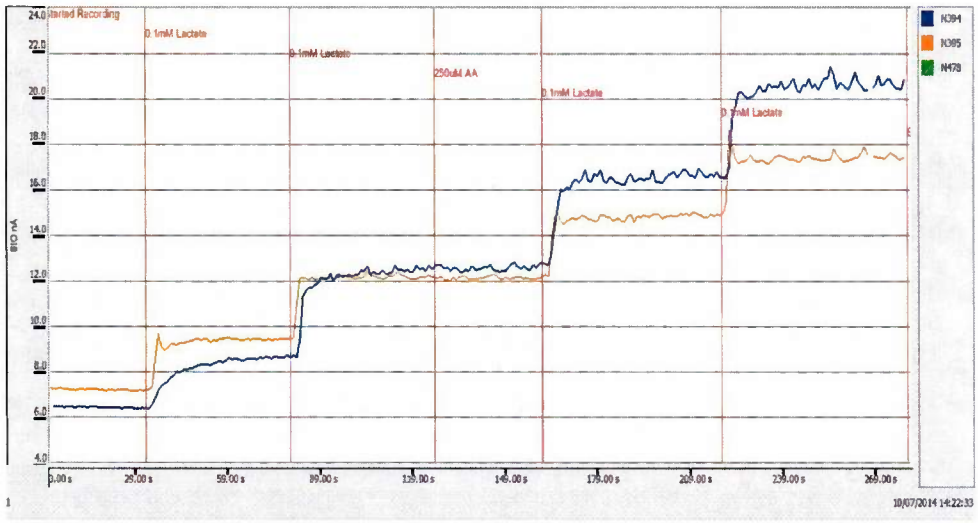


Figure 33 In vitro response of subcutaneous lactate biosensors.

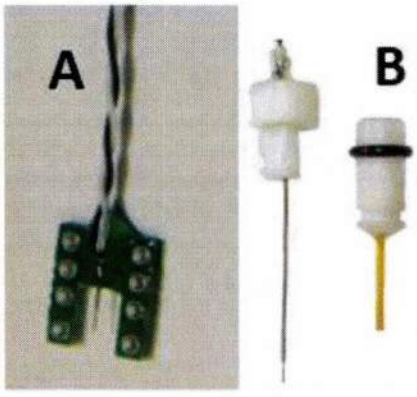


Figure 34 A) Physical substrate for a subcutaneous implantation. B) Typical intracerebral sensor device design (left) utilizing an intracerebral guide cannula (right).

Several *in vivo* experiments were performed – one with a biosensor made from the commercial enzyme, and one fabricated from MBP-Lox performed under anesthesia and

others in freely moving animals. In all cases, the biosensor was secured in place (Figure 35), covered by Tegederm film and the animal was fitted with a rat jacket. The biosensor was connected to a wireless 8164 Potentiostat and the animal was allowed to recover in its home-cage (Figure 36). A run-in period of at least 24 hours occurred.

In the first experiment, the animals were anesthetized under isoflurane and a needle was inserted in close proximity to the implanted biosensors. A bolus injection of lactate (0.5 g/mL lactate in 0.9% saline) was delivered to the implanted sensors under continuous isoflurane anesthesia. A response to the bolus injection was evident on both the Sekisui enzyme biosensor and the Pinnacle MBP-lactate oxidase enzyme sensor (Figure 37). During administration of the lactate solution some leakage occurred around the implant (through suture lines in skin and around actual implantation site) such that determination of the actual volume delivered to the sensor implant was not possible (in each case the intended administration volume was approximately 0.1 to 0.2 mL).

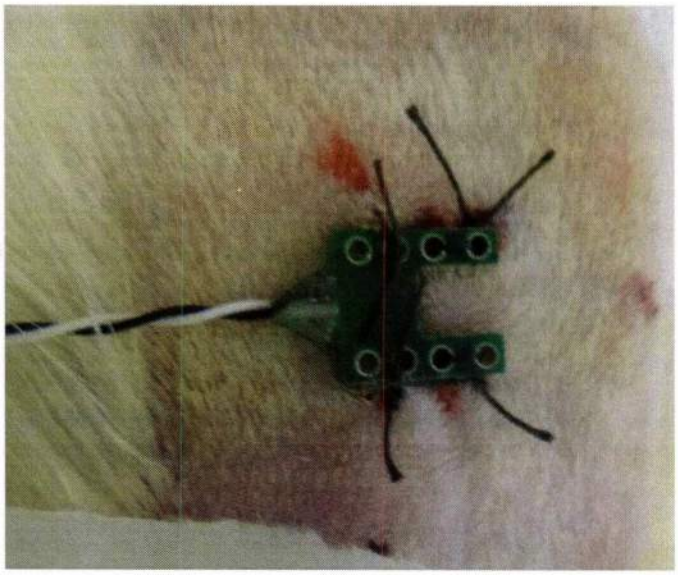


Figure 35 The subcutaneous lactate biosensor device secured to the skin with several sutures.

Figure 36 The complete monitoring package for the subcutaneous biosensor device.

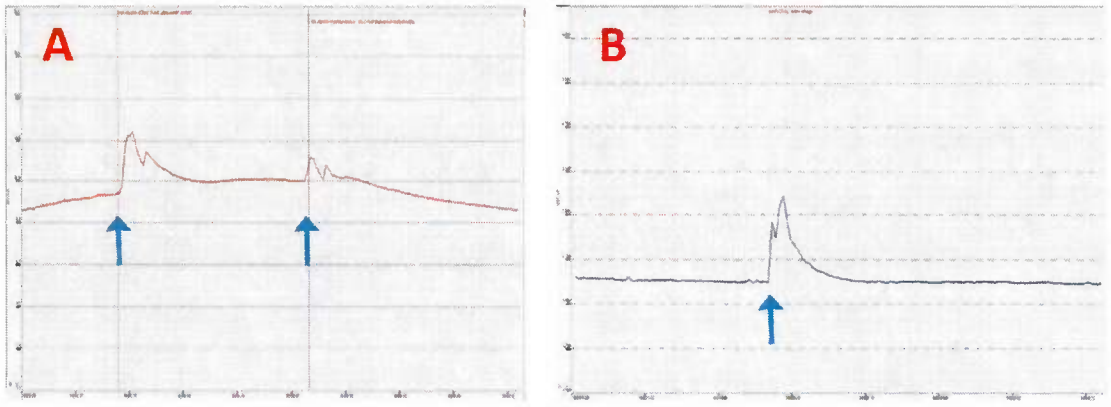


Figure 37 In vivo response of the subcutaneous lactate biosensors to local administration of lactate (0.5 g/mL in 0.9% saline). A: Sekisui enzyme biosensor (red trace). B: Pinnacle MBP-Lactate Oxidase biosensor (blue trace).

4.4 Histamine Oxidase Validation

The preliminary results for the MBP-zmPOA derived biosensors are shown in Table 3.

Table 13 Summary of MBP-zmPOA derived biosensors. The MBP-zmPOA was formulated in trehalose and had a unit activity of <2 U/mg

Sensor	*2uM Hist*	*2uM Hist*	*AA*	*2uM Hist*	*2uM Hist*	
*272	0.3	0.1	3.6	0.0	0.0	MBP-zmPOA
*274	0.2	0.2	3.3	0.0	0.0	MBP-zmPOA

We initially reported some success with biosensors developed using MBP-zmPaOX. This was inaccurate. There is an electroactive component to the histamine response that was not immediately recognized, and this confounded the initial testing. As an example of the effect see Figure 38 below. Diamine oxidase responds to histamine, but it is not specific to histamine. In this case, biosensors constructed with diamine oxidase show a clear stepwise response to the addition of histamine, but the bare platinum electrodes, and the electrodes with an inner membrane, but no oxidase also show a small response. That is, some component of the overall signal was derived from a non-enzymatic response to histamine.

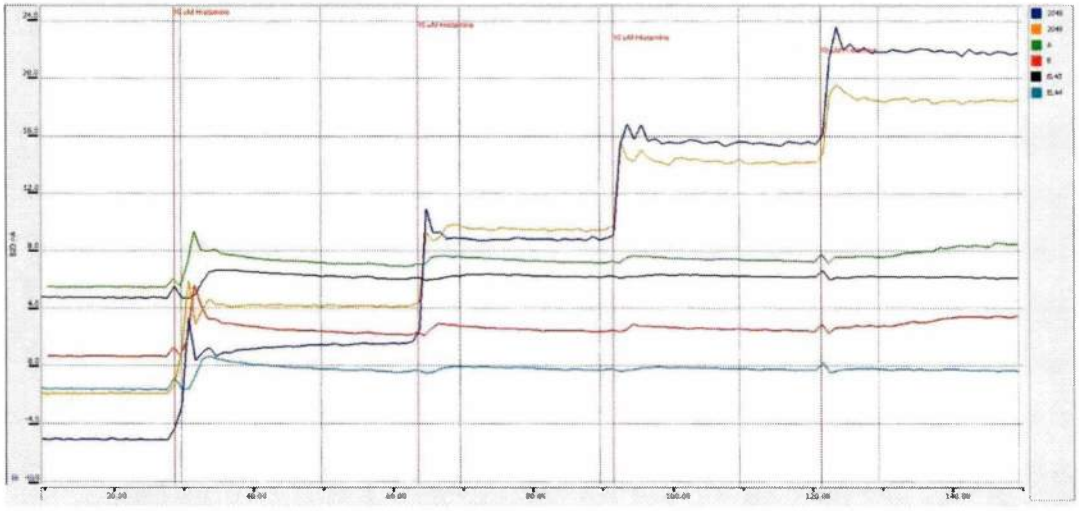


Figure 38 Histamine response of biosensors fabricated with diamino oxidase (blue, orange), bare platinum wire (red, green), and bare platinum with inner membrane but on enzyme (black, blue).

When the early data were analyzed with this interaction in mind. It was clear that the responses seen were in in the range of those expected from the non-enzymatic interaction.

Figure 39 below shows example traces of some histamine biosensors made using the best optimized histamine oxidase developed under this project when direct histamine to platinum reactions are eliminated. As can be seen, while this enzyme is active and it may be well suited to other applications, it does not demonstrate a significant response on this type of biosensor. For this reason, no additional *in vivo* testing was carried out.

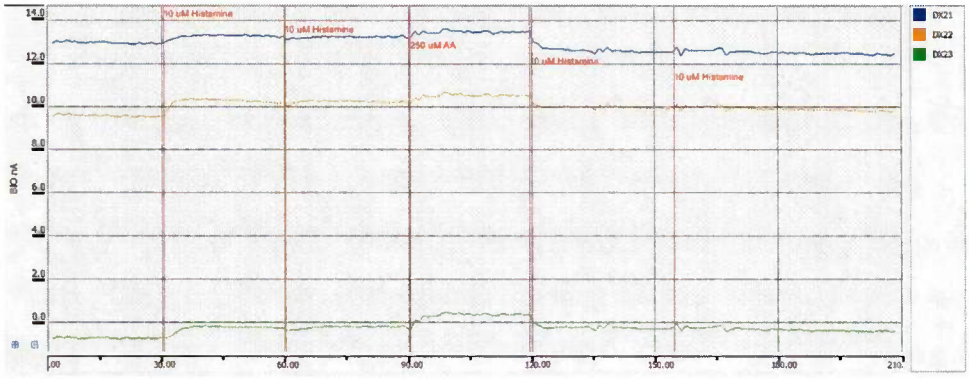


Figure 39 Summary response traces of histamine biosensors made using the currently optimized histamine oxidase

4.5 Cortisol Oxidase Validation

The cholesterol oxidase developed during this project is stable both in terms of temperature and detergents (Table 14). We were able to construct biosensors based on all of the cholesterol oxidase constructs that showed promising response.

Table 14 Summary of a range of cholesterol biosensors

		Cholesterol Added				
	Sensor	5uM	5uM	10uM	10uM	10uM
SUMO-COX1	*521	3.16	1.72	0.86	2.58	0
	*522	2.79	2.28	0.99	0.6	0.8
	*523	2.99	1.92	1.17	0.75	0.97
MBP-COX1	*526	0.21	1.34	0.5	0.46	0.71
	*527	0.19	0.25	0.53	0.26	0.44
	*528	0.2	0.25	0.54	0.42	0.38
MBP-COX2	*529	0.2	0.4	0.5	0.69	0.84
	*530	0.13	0.27	0.22	0.74	0.96
	*534	0.15	0.38	0.34	0.68	0.91

However, the response to cortisol remained low throughout the project. Cortisol biosensors were constructed, but as was the case with histamine, there is some direct action between the cortisol and platinum substrate. We believe that this interaction is the primary mechanism for the response shown in Figure 40. Some preliminary subcutaneous testing was performed (Figure 41, and Figure 42), but once it was clear that the primary response was likely due to direct cortisol/platinum interaction, no additional *in vivo* testing was carried out.

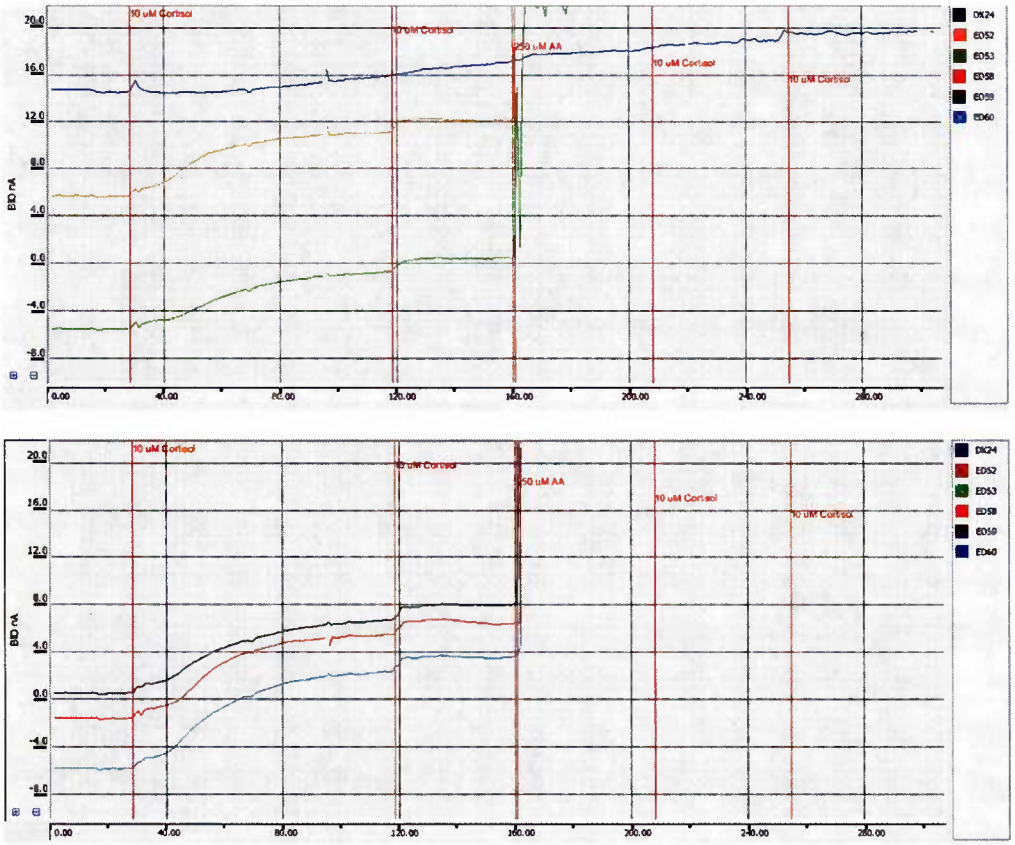


Figure 40 Summary response traces of cortisol biosensors made using the currently optimized cortisol oxidase. (a) Top traces using 1x loading of enzyme. (b) Bottom traces using 5x loading of enzymes

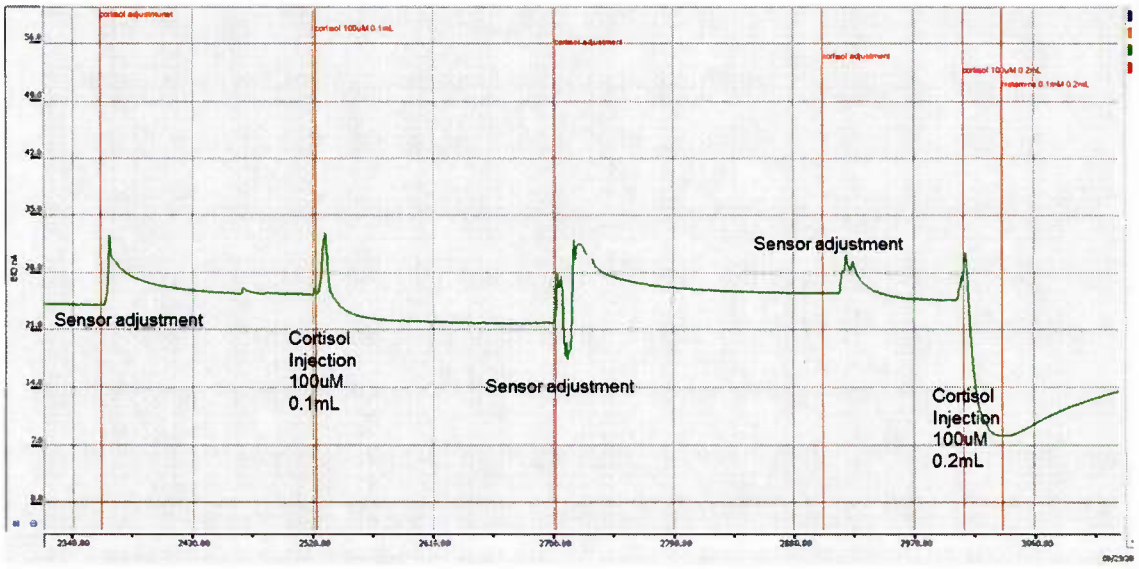


Figure 41 First experiment: Sub-cutaneous in vivo performance of a 5x-cortisol biosensor showing the response from multiple boluses of cortisol.

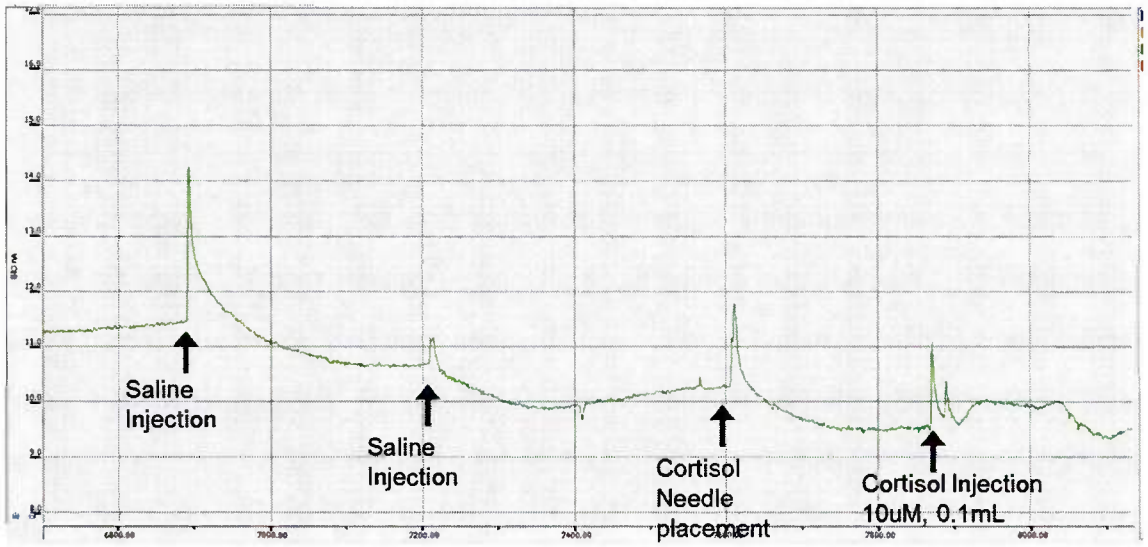


Figure 42 Second experiment: Sub-cutaneous in vivo performance of a 5x-cortisol biosensor showing the response to a cortisol bolus.

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